Mechanisms and evolution of prezygotic reproductive isolation in the parasitoid wasp genus *Nasonia*

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES
DER NATURWISSENSCHAFTEN (DR. RER. NAT.)
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vorgelegt von

Magdalena Mair

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Hall in Tirol, Österreich

im Jahr 2018

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Summary

Reproductive interference is a common phenomenon in nature. During reproductive encounters, females and males of different species frequently engage in interspecific reproductive behaviours resulting in fitness costs for either one or both of the two individuals. Reproductive interference usually involves mistakes made by the individuals in the process of species recognition during mate choice. These mistakes include, among others, the attraction of the wrong mating partner owing to signal interference, misdirected courtship owing to false male mate recognition, and interspecific copulation owing to mistakes in female mate discrimination. In insects, species recognition is largely based on chemical messengers, but may also involve acoustic or visual cues. The costs resulting from reproductive interference include the time and energy wasted in interactions with inadequate mating partners and the production of unfit or nonviable offspring. As a consequence of these costs, individuals that are able to avoid them should be favoured by natural selection. The evolution of strengthened prezygotic reproductive isolation mechanisms through natural selection is called reinforcement and includes a variety of mechanisms such as, among others, temporal or spatial divergence of mating, divergence in male reproductive behaviour, increased species discrimination and a shift in the messengers used during mate recognition.

In this thesis, I investigated the mechanisms and evolution of prezygotic reproductive isolation by reinforcement in species of the parasitoid wasp genus *Nasonia* (Hymenoptera, Pteromalidae). I show that the *Nasonia* species differ in various aspects of their reproductive behaviour and communication. The behaviour of males at the natal host patch differs profoundly among the *Nasonia* species and is characterised in detail in the two species *N. vitripennis* and *N. giraulti*. I further show that males of these two species use different chemical messengers to recognise conspecific mating partners. In addition, I show that females of *N. longicornis*, but not those of *N. vitripennis*, are able to adjust their mate acceptance behaviour in response to the actual presence or absence of heterospecific males. Finally, I investigated reinforcement directly by establishing artificial sympatry between the two naturally allopatric species *N. longicornis* and *N. giraulti*. I argue that species differences observed at present have likely evolved by reinforcement of prezygotic reproductive isolation in the past and discuss how divergent species characteristics likely help the species to co-exist in microsympatry in nature.

General introduction

Reproductive interference

Reproductive interference is a common phenomenon among animals living in sympatry (Gröning & Hochkirch, 2008) and has been reported in various insect taxa (Gröning & Hochkirch, 2008; Kishi, 2015; Shuker & Burdfield-Steel, 2017). It occurs when individuals of two species engage in reproductive behaviours which result in fitness costs for at least one of the two individuals (Gröning & Hochkirch, 2008; Shuker & Burdfield-Steel, 2017).

Reproductive interference often occurs between closely related species or species pairs which resemble in their sexual signals, courtship pattern or habitat preferences (Andrews et al., 1982; Hochkirch et al., 2006; van Gossum et al., 2007; Remnant et al., 2014) and results mostly from mistakes in species discrimination made by the individuals during reproductive encounters (Gröning & Hochkirch, 2008; Shuker & Burdfield-Steel, 2017). Mistakes in species discrimination occur for example during the attraction of mating partners by chemical (Andrews et al., 1982; Landolt & Heath, 1987; Groot et al., 2010), visual (Hochkirch et al., 2006) or acoustic (Gerhardt & Klump, 1988; Doherty & Howard, 1996) signals resulting in the attraction towards the wrong mating partners (signal interference), during male-female encounters resulting in misdirected courtship (Andrews et al., 1982; Singer, 1990; Gröning et al., 2007; Ben-David et al., 2009; Bath et al., 2012), or through mistakes in female mate discrimination during courtship resulting in interspecific copulation (Takafuji et al., 1997; Ben-David et al., 2009).

Depending on the stage in which mistakes are made, different costs may arise for the interacting individuals (Gröning & Hochkirch, 2008). Individuals being attracted to chemical, visual or acoustic signals released by heterospecific mates loose time and energy spent on movements towards the wrong mating partner (Gerhardt & Klump, 1988; Ardeh et al., 2004; Amézquita et al., 2011). Males that mistakenly court heterospecific females suffer from a loss of time and energy they could have spent in the courtship of conspecific females instead (van Gossum et al., 2007). Furthermore, the costs of misdirected courtship become particularly high in species in which courtship involves the transfer of nuptial gifts (Vahed, 1998). When females consent to interspecific copulation owing to incorrect female mate discrimination, energy is lost in both sexes by the waste of gametes, and by the production of unfit hybrids or

nonviable offspring (Takafuji et al., 1997; Orr & Presgraves, 2000; Hettyey & Pearman, 2003; Ben-David et al., 2009; Remnant et al., 2014; Shuker et al., 2015).

Reinforcement

As a consequence of the costs arising from reproductive interference, individuals that are able to avoid these costs should be favoured by natural selection, and mechanisms of prezygotic reproductive isolation should evolve between reproductively interfering species. This strengthening of prezygotic isolation through natural selection is called reinforcement (Butlin, 1987; Noor, 1999; Servedio & Noor, 2003; Servedio, 2004).

One means by which reproductive interference can be avoided is by a shift of the time or place where copulations usually happen (Higgie et al., 2000; Gröning et al., 2007; Urbanelli et al., 2014), or by the divergent evolution of reproductive behaviours exhibited by the interfering species, e.g. the evolution of increased mate discrimination (Liou & Price, 1994; Hudson & Price, 2014). The resulting pattern of divergent reproductive traits in areas of sympatry as a consequence of reinforcement is called reproductive character displacement (Howard, 1993).

Insects usually rely on chemical messengers to attract and recognise mating partners (Wyatt, 2014). To avoid chemical communication interference, closely related species that resemble in their reproductive behaviour therefore often use different chemical messengers in their sexual communication (Weiss, Hofferberth, et al., 2015; Weiss, Ruther, et al., 2015). In addition, when the presence of the interfering species varies considerably over time, it may be advantageous for individuals of these species to adjust their mate recognition and mate discrimination behaviour in a more plastic way, e.g. by a flexible learning or conditioning scheme (Irwin & Price, 1999; Kozak & Boughman, 2009; Crowder et al., 2010).

In nature, evidence that reinforcement has acted on a species pair in the past can be found when closely related species or species that resemble in some aspects of their reproductive behaviour differ in others, e.g. the chemical messengers used in sexual communication, or temporal or spatial differences in their mating behaviour. More reliable evidence for past reinforcement can be found in species pairs that occur in both allopatry and sympatry in different areas. In these species pairs, reproductive character displacement is expected in areas of sympatry, but should be absent in areas of allopatry (Gabor & Ryam, 2001). Furthermore, reinforcement can be studied by establishing experimental or artificial sympatry between two species that usually occur in allopatry in nature. This can be done by either controlled experiments in the lab or under semi-field conditions (Higgie et al., 2000; Urbanelli et al.,

2014), or by observing the reproductive behaviour of species that came into contact after the, either planned or accidental, anthropogenic introduction of individuals to new areas (D'Amore et al., 2009; Remnant et al., 2014). Although several studies have found evidence for the presence of reproductive interference and reinforcement in nature and some have highlighted their role in shaping the biogeographic distribution and co-existence of species, the evolution of reproductive isolation mechanisms and their role in speciation are not completely understood.

The genus *Nasonia*

The parasitoid wasp genus *Nasonia* Ashmead, 1904 (Hymenoptera, Pteromalidae) is an excellent model system for the study of the evolution of prezygotic reproductive isolation mechanisms. The chemical ecology of *Nasonia* is one of the best understood in insects (Ruther, 2013). For decades, *Nasonia vitripennis* (Walker, 1836) (*Nv*) has served as a model for parasitoid wasp behaviour and chemical communication (van den Assem, 1996; Ruther, 2013), and the knowledge of the ecology and behaviour of the other three *Nasonia* species, *N. giraulti* Darling, 1990 (*Ng*), *N. longicornis* Darling, 1990 (*Nl*) and *N. oneida* Raychoudhury & Desjardins, 2010, is growing steadily (e.g., Leonard & Boake, 2006; Giesbers et al., 2013; Ruther et al., 2014). In addition, the *Nasonia* species show a distribution which allows for the study of reinforcement by both, a comparison of the different species' reproductive behaviour and the establishment of artificial sympatry between naturally allopatric species (Darling & Werren, 1990; Raychoudhury, Desjardins, et al., 2010). Finally, the availability of the whole genome sequences of all *Nasonia* species together with the model status of the genus in various fields of biological research opens up future possibilities for the study of the molecular mechanisms underlying the evolution of pre-zygotic reproductive isolation in general (Gadau et al., 2008; Werren et al., 2010). Furthermore, when brought into a wider context using a comparative approach with other parasitoid wasps, knowledge of reinforcement in *Nasonia* may give further insights into the role that the evolution of divergent reproductive traits plays in speciation in general.

Thesis outline

In this thesis, some aspects of reproductive interference and behavioural divergence among the species of the parasitoid wasp genus *Nasonia* are investigated and the species differences are discussed in the context of the evolution of pre-zygotic reproductive isolation by reinforcement (chapters 3-5). In addition, reinforcement by increased female mate discrimination is investigated directly by using an artificial sympatry approach (chapter 6).

Chapter 2 gives a detailed introduction to the chemical ecology of the genus *Nasonia* following the wasps' life cycle from emergence to oviposition. Particular focus is laid on the chemical messengers used by the wasps at different stages of their adult life. In addition, biosynthetic pathways are depicted where known, differences between the *Nasonia* species are highlighted, ecological and evolutionary implications are discussed, and insights into the wasps' olfactory perception and learning abilities are summarised.

In chapter 3, the differences between *Nv* and *Ng* male behaviour at the natal host patch are characterised and the implications of these differences in microsympatry are discussed. *Nv* males are territorial and mate outside the host after emergence, whereas *Ng* males are not territorial and mate inside the host prior to emergence. Although almost all *Ng* females emerge from the host mated, *Ng* males produce and deposit an abdominal sex pheromone outside the host which is attractive to females. The adaptive value of *Ng* marking behaviour in the presence of the microsympatric species *Nv* in nature is discussed.

In chapter 4, differences in mate discrimination and differences in the chemical messengers used during male mate recognition are investigated in the species *Ng* and *Nv*. While *Nv* males rely solely on cuticular hydrocarbons to recognise females, *Ng* males use additional chemical messengers, presumably more polar cuticular lipids, to recognise conspecific mates. A past shift from cuticular hydrocarbons to other female messengers in *Ng* is discussed.

In chapter 5, the plasticity of female mate discrimination in *Nl* and *Nv* is investigated. When having been unsuccessfully courted by a heterospecific male, *Nl* females are subsequently more reluctant to mate with a conspecific. This behavioural plasticity has not been observed in *Nv* females, however. The adaptive value of the flexible adjustment of mate acceptance in *Nl* in response to the actual absence or presence of *Nv* in the environment at a given time and place is discussed.

In chapter 6, reinforcement of reproductive isolation by increased mate discrimination is investigated in *Nl* females by rearing *Nl* wasps in artificial microsympatry with males of the naturally allopatric species *Ng*. Although the two species most likely interfered reproductively in artificial sympatry, reinforcement by increased female mate discrimination did not evolve. The lack of reinforcement by increased female mate discrimination in the experiment is discussed.

Finally, in chapter 7, the results of the described studies are brought into a wider scientific context and possible future directions of research are discussed.

2. Chemical ecology of the parasitoid wasp genus *Nasonia* (Hymenoptera, Pteromalidae)

Magdalena M. Mair and Joachim Ruther

Author contributions: Writing: Original Draft Preparation, M.M.M.; Writing: Review & Editing, M.M.M. and J.R.; Visualization, M.M.M and JR.

ABSTRACT

The use of chemical cues and signals is essential for communication processes in insects. Wasps of the genus *Nasonia* (Hymenoptera, Pteromalidae) are gregarious parasitoids that lay their eggs into puparia of cyclorrhaphous flies. During their life cycle, various kinds of semiochemicals are used: (1) a male abdominal sex pheromone that attracts females and induces site fidelity in males, (2) a female-derived contact sex pheromone eliciting courtship behaviour in males, (3) an oral male aphrodisiac eliciting receptivity signalling in females and causing a switch in the females' olfactory preferences, (4) chemicals derived from host habitat and host puparia used in olfactory host finding by female wasps, and (5) chemicals used by females to assess the quality and parasitisation status of potential hosts. We review the literature on the chemical ecology of *Nasonia* spp. following the wasps' life cycle from emergence to oviposition. We depict biosynthetic pathways where available, discuss ecological implications, highlight differences among *Nasonia* species, summarise insights into their olfactory perception and associative learning abilities, and point out gaps in our understanding of the chemical ecology of these parasitoids to be addressed in future studies.

INTRODUCTION

Chemicals are highly important to insect life (Cardé & Baker, 1984; Symonds & Elgar, 2008; Wyatt, 2014). They are used in various kinds of communication processes in several stages of the insects' life cycles. Since the identification of bombykol, the sex pheromone used by females of the silkworm moth *Bombyx mori* for long-range attraction of males (Butenandt et al., 1959), and the following establishment of the field of chemical ecology in the early 1960s, particular focus has been laid among other topics on the identification of chemical messengers used by insects to find mating partners and locate adequate foraging and oviposition sites (Greenfield, 1981; Cardé & Baker, 1984; Renwick, 1989; Landolt, 1997; Pichersky & Gershenzon, 2002; Wyatt, 2014). Setting out for a specific destination in an often complex environment usually necessitates different stages of searching behaviour. This includes the location of an adequate habitat using long-range volatiles and narrowing down the search to ever smaller spatial scales until finally making use of non-volatile substances to recognise mating partners or assess the quality of a food item or host by direct contact (Vinson, 1976). On the long range, the process of mate finding often involves the release of highly volatile sex pheromones by one sex and the attraction to these pheromones by individuals of the other sex (Greenfield, 1981). On the short range, males usually recognise females based on chemical messengers distributed over the females' cuticle (Lockey, 1988; Blomquist & Bagnères, 2010) and courtship frequently involves the transfer of a species-specific sex pheromone which allows females to assess not only the species of the courting male but also its quality (Birch & Hefetz, 1987; Scott et al., 1988).

In parasitic wasps, chemical messengers are used in a plethora of different situations. Mating partners encounter at aggregation sites by means of anemotaxis along gradients of volatile aggregation pheromones which are released by the aggregating wasps (e.g., Mohamed and Coppel 1987). In other species, mates are attracted by volatile sex pheromones released by either females (Quicke, 1997) or less frequently males (Cônsoli et al., 2002; Ruther et al., 2007). Female-derived cuticular lipids elicit courtship behaviour in males (e.g., Sullivan 2002) and during courtship, aphrodisiacs are transferred from the male's antennal or oral glands to the female's antennae (e.g., Ruther et al. 2010; Weiss, Ruther, et al., 2015). Mated females use host-associated chemicals to locate adequate hosts (Vinson, 1976) and avoid competition at oviposition sites by means of marking pheromones released by ovipositing females (Nufio et al., 2001). In the context of intraspecific competition, territorial markings are applied by males (Mair & Ruther, 2018) and

appeasement pheromones are released by females having lost in contest situations (Goubault et al., 2006).

For decades, *Nasonia vitripennis* has served as a model for parasitic wasp behaviour (Whiting 1967; van den Assem 1986). Since the identification of the three other *Nasonia* species (Darling & Werren, 1990; Raychoudhury, Desjardins, et al., 2010), research interests have been broadened addressing now a wide variety of questions related to various different research fields including, among others, genetics, evolution, development, neurobiology and chemical ecology (Gadau et al., 2008; Werren & Loehlin, 2009; Werren, Richards, Desjardins, Niehuis, Gibbs, et al., 2010; Schurmann et al., 2012; Ruther, 2013; Groothuis & Smid, 2017; Tappert et al., 2017). *Nasonia* wasps are easy to breed in the lab, easy to handle and due to their species structure and distribution pattern form an exceptional model system for the study of the evolution of species-specific chemical communication and other reproductive isolation mechanisms. Furthermore, the availability of the whole genome sequences of all *Nasonia* species opens up valuable opportunities to investigate biosynthetic pathways in detail and get new insights into insect chemical perception in general.

The genus *Nasonia*

Wasps of the genus *Nasonia* (Hymenoptera, Pteromalidae) are parasitoids of pupae of various cyclorrhaphous flies (Diptera) found in nests of hole-breeding birds and on rotting carcasses (Darling & Werren, 1990; Raychoudhury, Desjardins, et al., 2010). The wasps are ectoparasitic and lay their eggs onto the surface of the fly pupa inside the fly puparium. Larvae feed on the host from the outside of the host body, pupate inside the host puparium and emerge from the puparium after eclosion. *Nasonia* wasps are gregarious, i.e. females lay more than one egg per host, and frequently several females lay their eggs into the same fly puparium (Grillenberger et al., 2008). Courtship and copulation happen at the natal host patch after emergence and females disperse after mating (King et al., 2000; Grillenberger et al., 2008; Ruther et al., 2014).

The genus *Nasonia* consists of four species. *Nasonia vitripennis* (*Nv*; (Walker 1836), the species most intensely studied, is cosmopolitan and occurs sympatrically with all other *Nasonia* species (Raychoudhury, Grillenberger, et al., 2010). *Nasonia longicornis* (*Nl*; (Darling and Werren 1990) inhabits the West of North America, whereas *N. giraulti* (*Ng*; Darling and Werren 1990) is restricted to the East and *N. oneida* (*No*; (Raychoudhury et al. 2010a) is merely known from two locations in New York State, co-occurring with both, *Ng* and *Nv*. Sympatric *Nasonia* species are frequently found on the same host patch and may

even develop in microsympatry, i.e. within the same host individual (Darling & Werren, 1990; Grillenberger, van de Zande, et al., 2009; Raychoudhury, Desjardins, et al., 2010; Raychoudhury, Grillenberger, et al., 2010). In the natural environment, it is therefore likely that adult females and males of two sympatric species encounter and interfere on shared host patches. All *Nasonia* species, except for the species pair of *Ng* and *No*, are reproductively isolated by *Wolbachia*-induced cytoplasmic incompatibility resulting in paternal chromosome loss after fertilisation with heterospecific sperm (Breeuwer & Werren, 1990; Bordenstein et al., 2001). Females having mated with heterospecific males are not able to produce hybrid offspring. Instead, because *Nasonia* like all hymenopterans are haplodiploid, eggs fertilised by heterospecific sperm either die or develop into male offspring similar to unfertilised eggs (Breeuwer & Werren, 1990; Tram et al., 2006). As females of *Nasonia* usually mate only once during their lifetime, interspecific copulations are particularly costly for them (Liou & Price, 1994). Mechanisms involving adaptations in chemical communication have therefore evolved between *Nasonia* species to avoid and counteract the risks and costs of copulating with the wrong partner (e.g. Giesbers et al. 2013; Ruther et al. 2014).

The chemical communication system, particularly that of *Nv,* is one of the best understood in insects. Studies on semiochemicals used by *Nv* have revealed pheromones and allelochemicals which are highly important for the wasps in almost all stages of their life: Males use sex pheromones to scent mark territories and arrest females after emergence from the host (e.g. Ruther et al. 2007). They use female-derived contact sex pheromones to recognise potential mates (e.g. Steiner et al. 2006). During courtship, the male mounts the female and performs specific courtship movements coupled with the transfer of an aphrodisiac from the male's cephalic glands to the female's antennae to induce female receptivity (e.g. van den Assem et al. 1980b). After mating, females use host habitat cues to locate new host patches (e.g. Frederickx et al. 2013) and are able to assess host quality and the status of pre-parasitisation by other females through chemical inspection with their ovipositors (e.g. King and Rafai 1970; Blaul and Ruther 2011; for a summary of the semiochemicals used in *Nasonia* see Figure 1 and Table 1).

In this article, we review literature on the chemical ecology of all four *Nasonia* species following the wasps' life cycle from emergence to oviposition. Focus is laid on chemical messengers used by the wasps at different stages of their lives including, where available, information on biosynthetic pathways and ecological implications. Recent studies have

revealed that the different *Nasonia* species differ in far more aspects of chemical communication and behavioural strategies than previously thought (Leonard & Boake, 2006; Niehuis et al., 2013; Ruther et al., 2014; Giesbers et al., 2016; Mair et al., 2017; Mair & Ruther, 2018). After discussing information available for *Nv,* differences to the other three *Nasonia* species are thus highlighted where they are known. In addition, we give a short overview about what is known of the wasps' olfactory associative learning abilities and olfactory perception, including antennal morphology, sensory sensillae and the genetic basis of chemosensory receptors and odorant binding proteins.

(1) TERRITORIALITY AND MATE ACQUISITION – THE MALE ABDOMINAL SEX PHEROMONE

Males of *Nv* emerge protandrously, i.e. they emerge from the host prior to females by chewing an emergence hole into the fly puparium (Giesbers et al. 2016). The first male emerging from the host usually builds up a territory on the host which is defended aggressively against all other males emerging later on or intruding from nearby hosts (van den Assem, Gijswijt, et al., 1980; van den Assem, 1986; Leonard & Boake, 2006; Mair & Ruther, 2018). Subordinate males stay close to the territory and interfere with the territorial male regularly by challenging its position on the host. The territorial structure of a group can persist over longer time periods, but territoriality becomes increasingly unstable with increasing group size, finally resulting in scramble competition for females emerging from the host later (van den Assem et al. 1980a). Emerging females are mounted and courted by the first male they encounter. By dominating the position on the host from which females are about to emerge, a territorial male should thus get prioritised access to copulations with females. However, observations under semi-natural conditions have been unsuccessful in demonstrating a fitness benefit for territorial males over subordinate males in the lab (Mair & Ruther, 2018). In contrast to territorial males, subordinate males gain copulation opportunities by following an alternative reproductive strategy (Mair & Ruther, 2018). They frequently mount the female together with the territorial male, position themselves on the female's abdomen, sneak in when the female signals receptivity and copulate with the female instead of the courting territorial male. In addition, when two or more females emerge in a quick succession, it may happen that the territorial male is still occupied with courting the

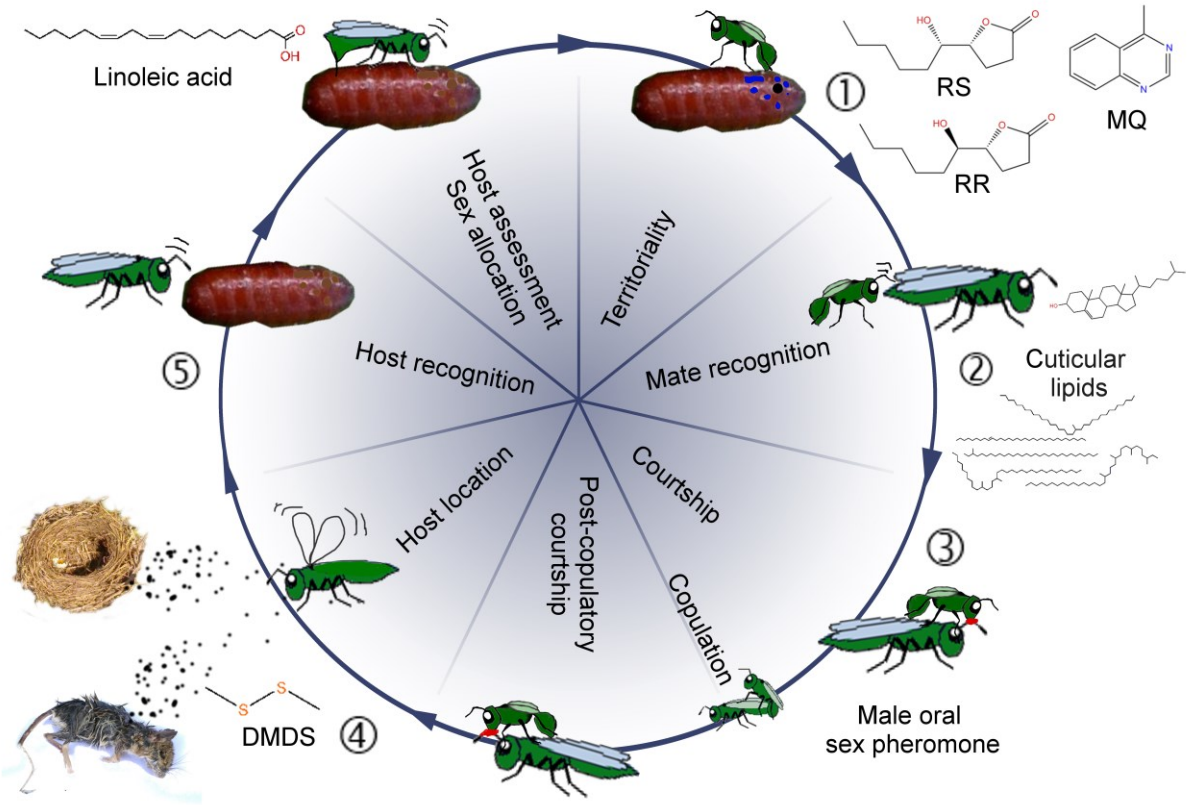

Figure 1 Life cycle of *Nasonia* with emphasis on the different stages at which semiochemicals are involved. (1) Males apply an abdominal sex pheromone to the natal host and its surroundings to attract and arrest emerging females. (2) Males recognise females based on the females' cuticular lipids. (3) During courtship, a male oral sex pheromone is transferred to the female's antennae, females discriminate between conspecific and heterospecific mating partners and post-copulatory courtship induces a switch in the females' receptivity. (4) Females find new hosts based on olfactory cues. (5) Chemical messengers are most likely involved during host recognition, host assessment and sex allocation (according to local mate competition theory). RR: (4*R*,5*R*)-5-hydroxy-4-decanolide, RS: (4*R*,5*S*)-5-hydroxy-4-decanolide, MQ: 4-methylquinazoline, DMDS: dimethyldisulphide.

first female when the second female emerges, giving nearby subordinate males the opportunity to court and mate with the female themselves (Mair & Ruther, 2018).

Territoriality in *Nv* is accompanied by the application of pheromonal scent marks deposited by the territorial male on the surface of the host and areas nearby by performing dapping and streaking movements with its abdomen over the substrate, a behaviour termed 'abdomen dipping' (Barrass, 1969; Steiner & Ruther, 2009b; Ruther et al., 2011; Mair & Ruther, 2018). Marking behaviour is intensified after contact to females and exhibited particularly often after successful copulation (van den Assem, 1986; Steiner & Ruther, 2009b). Territorial males show marking behaviour more often than subordinate males (Mair & Ruther, 2018). Nevertheless, subordinate males exhibit marking behaviour outside the

central territorial area, likely at spots where they have previously encountered and eventually copulated with a female (Steiner & Ruther, 2009b; Mair & Ruther, 2018). The pheromonal markings are highly attractive for both females and males, and males do not distinguish between their own markings and those laid by their competitors (van den Assem, 1986; Ruther et al., 2011). By eliciting site fidelity in both sexes, the pheromone prevents virgin females from moving away from the host after emergence and enables males to locate spots where females have been encountered before, either by themselves or by other males. Once deposited, the pheromone markings are attractive for ca. 2-3 h and a chemical basis for the attractiveness has been suggested already in 1980 (van den Assem, Jachmann, et al., 1980; Steiner & Ruther, 2009b). The components of the abdominal marking pheromone have been identified as the two stereoisomers (4*R*,5*S*)- and (4*R*,5*R*)-5-hydroxy-4-decanolide (RS and RR, respectively) and the minor pheromonal component 4-methylquinazoline (MQ; Ruther et al. 2007, 2008, 2011; Steiner and Ruther 2009). All three components are synthesised in the male rectal vesicle and are absent in females (Abdel-Latief et al., 2008). Males are attracted only by MQ whereas RS and RR do not elicit any specific behavioural responses (Ruther et al., 2011). In contrast, females are attracted by RS alone which is synergised by both RR and MQ (Ruther et al., 2007, 2008; Steiner & Ruther, 2009b; Niehuis et al., 2013). In bioassays, the strongest attraction of females has been observed when all three components were presented together (Niehuis et al., 2013). In addition, the pheromone response of females is concentration dependent, with females preferring higher deposited amounts over lower ones (Ruther et al., 2009; Blaul & Ruther, 2011). After copulation, however, females are no longer attracted to the abdominal sex pheromone, become restless instead and switch to host-seeking behaviour (Ruther et al., 2007, 2010, 2014; Steiner & Ruther, 2009a).

RS and RR occur typically in the rectal vesicle at a 2:1 ratio and sex pheromone titres as well as the amount of pheromone actually deposited by males are correlated with male body size and with male mating history (Ruther et al., 2009; Blaul & Ruther, 2012). Larger males produce and deposit more pheromone (up to 1 µg HDL, i.e. RS plus RR) than smaller males (Blaul & Ruther, 2012), and male pheromone titres decrease with repeated marking activity following after each copulation (Ruther et al., 2009; Blaul & Ruther, 2012). Markings deposited by multiply mated males are thus less attractive to females than markings deposited by virgin males. The fact that significant sperm depletion occurs already

Table 1 Semiochemicals used by the four *Nasonia* species, *N. vitripennis* (*Nv*), *N. giraulti* (*Ng*), *N. longicornis* (*Nl*) and *N. oneida* (*No*). +: substance(s) present in or used by the respective species, -: substance(s) absent, bold: species in which the use and function of the respective substance(s) has been shown in experimental bioassays, ?: no data available.

			Nv	***Ng***	***Nl***	***No***	**Semiochemical class**	**Source**	**Function**
territoriality and courtship	male abdominal sex pheromone	RS[1]	**+**	**+**	**+**	?	sex pheromone marking pheromone	rectal gland	female attractant
		RR[2]	**+**	**-**[3]	**-**[3]	?	sex pheromone marking pheromone	rectal gland	female attractant synergist of RS
		MQ[4]	**+**	+	+	?	sex pheromone marking pheromone	rectal gland	male arrestment synergist of RS
	CHCs[5] CLs[6]		**+**	**+**	**+**	**+**	sex pheromone contact pheromone	distributed over the cuticle	species and sex recognition
	male oral sex pheromone		**+**	?	?	?	sex pheromone	mandibular gland?	triggers receptivity in females pheromone switch receptivity switch switch to host-seeking behaviour?
host finding	DMDS[6]		**+**	?	?	?		host habitat (decaying meat)	intermediate distance travel
	birds' nest odours		**+**	?	?	?		host habitat (birds' nests)	intermediate distance travel
host quality assessment	pupal odours		?	?	?	?	kairomones	host pupa	discrimination of host species?
	linoleic acid		**+**	?	?	?	kairomone	host pupa	indicates high quality of a host
	venom/ venom-induced changes in haemolymph		**+**	?	?	?	pheromone/ allelochemically induced kairomone	venom gland/ host pupa	assessment of parasitisation status of a hosts

[1](4*R*,5*S*)-5-hydroxy-4-decanolide, [2](4*R*,5*R*)-5-hydroxy-4-decanolide, [3] RR found in traces,
[4]4-methylquinazoline, [5]cuticular hydrocarbons, [6]cuticular lipids, [6]dimethyldisulfide.

after seven consecutive matings indicates that pheromone quantity may be used by females during mate choice as an honest signal of male fertility (Ruther et al., 2009). Even if having moved away from the territory when mounted on the female's back during courtship and copulation, territorial males usually return to their territory before intensifying marking activities (Mair & Ruther, 2018). Instead of applying new pheromonal spots elsewhere, they thus strengthen the signal of their earlier pheromonal deposits by adding further amounts of the pheromone. Females attracted to stronger pheromonal signals are therefore also attracted to reproductively and territorially successful males.

Further evidence that the amount of pheromone is indeed an honest indicator of male quality comes from studies on the biosynthetic pathway of the major pheromone component HDL (Figure 2). A chemical sexual signal can become honest, if its production is costly for the producer (Zahavi, 1975; Johansson & Jones, 2007). Often pheromone production involves biosynthetic pathways connected to other important metabolic functions effecting, for example, the individual's immunological defence against pathogens (Rantala et al., 2003) or the production of gametes (Thomas & Simmons, 2009). Linoleic acid ((9*Z*,12*Z*)-octadeca-9,12-dienoic acid, LA), a polyunsaturated fatty acid (PUFA), is needed for the production of sperm in animals (Wathes et al., 2007). Stable isotope labelling experiments revealed that LA is also a precursor for the biosynthesis of HDL in *Nasonia* males (Blaul & Ruther, 2011). This indicates a trade-off between abdominal sex pheromone production and the production of sperm. Consistently, males emerging from hosts artificially enriched in LA produce both larger amounts of sperm and larger amounts of HDL (Blaul & Ruther, 2011).

Recent studies on the pheromone biosynthesis revealed that *Nv* has a Δ12-desaturase enabling them to synthesise LA from oleic acid (OA; Blaul et al. 2014; Semmelmann et al., unpublished data). Wang et al. (2015) found the predicted desaturase gene Nasvi2EG017727 to be 800-fold higher expressed in *Nv* males and functional characterisation of the gene product subsequently revealed that it has in fact Δ12-desaturase activity and is expressed in the male pheromone gland (Semmelmann et al., unpublished data). The fact that *Nv* males are capable of synthesising LA by themselves challenged the postulated importance of LA as a limited resource. However, a recent study (Brandstetter & Ruther, 2016) revealed that males albeit synthesising LA still benefit from the dietary uptake of LA during larval development. Males reared on LA enriched hosts were able to produce significantly higher amounts of HDL than males reared on hosts enriched in OA indicating that the conversion of OA into LA is a costly process (Brandstetter & Ruther, 2016). The next step of the HDL

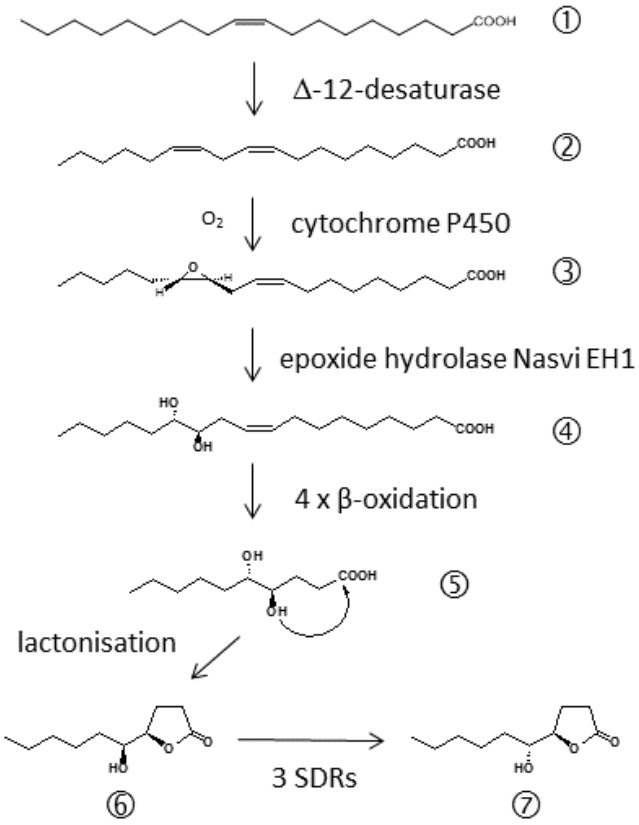

Figure 2 Biosynthetic pathway of the major *Nasonia* male abdominal sex pheromone component 5-hydroxy-4-decanolide (HDL). (1) Oleic acid, (2) linoleic acid, (3) 12,13-epoxy-(9*Z*)-octadecenoic acid, (4) 12,13-dihydroxy-(9*Z*)-octadecenoic acid, (5) 4,5-dihydroxydecanoic acid, (6) (4*R*,5S)-5-hydroxy-4-decanolide (RS), (7) (4*R*,5*R*)-5-hydroxy-4-decanolide (RR).

biosynthesis is an epoxidation of LA to 12,13-epoxy-(9*Z*)-octadecenoic acid. This conversion is typically catalysed by cytochrome P450 enzymes (CYP450; Oliw 1994). CYP450 genes are highly abundant in the *Nv* genome (Oakeshott et al., 2010), but the question which of the 92 candidate genes is involved in the pheromone biosynthesis needs further investigation. The next step of the HDL biosynthesis is the hydrolysis of 12,13-epoxy-(9*Z*)-octadecenoic acid to 12,13-dihydroxy-(9*Z*)-octadecenoic acid by the epoxide hydrolase which is encoded by the gene Nasvi-EH1 (Abdel-Latief et al., 2008). Gene expression experiments using *in situ* RT-PCR suggested that this step occurs in the rectal papillae, twins of secretory organs adjacent to the rectal vesicle (Davies & King, 1975). Four steps of chain shortening by β-oxidation and lactonisation of the resulting 4,5-

dihydroxydecanoic acid then eventually leads to RS. The second HDL stereoisomer RR is produced in *Nv* males by epimerisation of RS using short-chain dehydrogenases/reductases (SDRs) encoded by the three genes NV10127, NV10128, and NV10129 positioned on chromosome 1 (Niehuis et al., 2013; Ruther et al., 2016). The sequences of the *Nasonia* SDR genes are highly similar to those of enzymes catalysing the deactivation of prostaglandins which serve various hormonal functions in insects (Stanley, 2006). This suggests that the SDRs epimerising RS to RR in *Nv* have evolved secondarily from these enzymes by gene duplication and neofunctionalisation (Niehuis et al., 2013; Ruther et al., 2016).

The *Nasonia* species differ profoundly in the behaviour they exhibit at the natal host patch: while virtually all females of *Nv* emerge from the host puparium as virgins, almost all females of *Ng* mate inside the host prior to emergence (Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013; Ruther et al., 2014), and within-host-mating (WHM) rates in *Nl* and *No* lay between those of *Nv* and *Ng* (Leonard & Boake, 2006; Giesbers et al., 2013). Giesbers et al. (2016) suggested that the high WHM rate in *Ng* results from the fact that *Ng* males refrain from chewing an exit hole into the puparium and thus impede the emergence of virgin females. Consistently, in *Ng*, females emerge prior to males (Mair & Ruther, 2018). Instead of building up territories, *Ng* males readily disperse from the host after emergence and engage less often in aggressive interactions than males of *Nv* (Leonard & Boake, 2006; Mair & Ruther, 2018). Nevertheless, *Ng* males produce similar amounts of HDL as males of *Nv* (Ruther et al., 2014) and use the abdominal sex pheromone to mark the substrate in the surroundings of the natal host patch (Mair & Ruther, 2018). Mair and Ruther (2018) suggested that *Ng* marking, although useless when all females are already mated, is of adaptive importance in microsympatry with *Nv*. When wasps of *Nv* and *Ng* develop within the same host individual, an increased number of *Ng* females emerges as virgins because *Nv* males chew an exit hole into the puparium through which virgin *Ng* females can escape (Giesbers et al., 2016). In these situations, *Ng* males marking the substrate with abdominal sex pheromone may be able to attract and copulate with these unmated females (Mair & Ruther, 2018). Future observations of wasps emerging naturally from hosts multiparasitised by both *Nv* and *Ng* could give valuable insights into the dynamics occurring between these two species at the natal host patch in microsympatry.

A further difference between the *Nasonia* species concerns the composition of the marking pheromone. The male abdominal sex pheromone of *Nl, No* and *Ng* as well as the one of the closely related species *Trichomalopsis sarcophagae* consists of RS and MQ, lacking significant amounts of the third component RR (Niehuis et al., 2013). This suggests

that RS/MQ is the ancestral pheromone composition and RR has evolved in *Nv* as an adaptation to avoid interspecific mating caused by signal interference in areas of sympatry. Consistent with this hypothesis, RR has no effect on the pheromone response of *Ng* females while it synergises the response to RS in *Nv* females (Niehuis et al., 2013). Strikingly, genes encoding the SDRs which catalyse the epimerisation of RS to RR are also present in the genome of *Ng* and *in vitro* assays showed that they are also capable of catalysing the epimerisation albeit with a decreased efficiency. However, proteomic analyses of the pheromone glands showed a much higher expression in *Nv* suggesting that differential SDR gene expression underlies the pheromone difference between Nv and the other *Nasonia* species (Ruther et al., 2016).

Males of *No* produce less HDL than males of *Ng* and three quantitative trait loci (QTL; on chromosomes 1, 4 and 5 respectively) have been identified that are significantly correlated with male pheromone quantity in these two species (Diao et al., 2016). However, the functional characterisation of genes at these loci as well as the ecological implications of this difference in pheromone quantity necessitates further investigation. The behaviour of *No* at the natal host patch is largely unknown. It would be interesting to study whether HDL production and the release of the abdominal sex pheromone are further correlated to different behavioural strategies at the natal host patch, e.g. rendering pheromone deposition by *No* males less important than in the other *Nasonia* species. In addition, the amount of HDL produced by males of *Nl* has not been investigated so far. *Nl* is characterised by relatively low WHM rates of about ten percent (Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013) and engage in aggressive interactions on the host after emergence (Leonard & Boake, 2006) indicating that *Nl* males may exhibit territorial behaviour similar to that of *Nv* males. A detailed comparative study of male and female behaviour of the different species at the natal host patch coupled with behavioural bioassays using multiparasitised hosts may prove valuable for the understanding of how differences in the pheromone communication at the natal host patch have evolved and how the different *Nasonia* species are able to coexist in areas of microsympatry.

(2) MALE MATE RECOGNITION – FEMALE DERIVED CONTACT SEX PHEROMONES

When encountering another individual, males have to decide whether or not the individual is a possible mating partner to appropriately adjust subsequent decisions. In *Nv*, females are typically followed and courted by the males, whereas male competitors are either attacked or dominated and chased off (van den Assem, Gijswijt, et al., 1980). In addition, as a measure of prezygotic reproductive isolation, males should avoid courting heterospecific females or at least prefer conspecific over heterospecific ones if costs imposed by courting and copulating with the wrong mating partner are considerably high. In insects, male courtship is often induced by chemical messengers distributed over the females' cuticle (Lockey, 1988; Howard & Blomquist, 2005; Blomquist & Bagnères, 2010; Wyatt, 2014). These chemical messengers can be washed off using nonpolar solvents such as hexane or pentane which indicates that the chemical messengers eliciting courtship behaviour are cuticular lipids (CLs). One major class of CLs are the cuticular hydrocarbons (CHCs) which primarily function as a protection shield against desiccation (Lockey, 1988; Gibbs, 1998) and are known to play important roles in recognition processes in a wide variety of insect taxa (Singer, 1998; Blomquist & Bagnères, 2010). CHCs are the most abundant CLs found on the insects' cuticular surface and are easily detectable and identifiable by gas chromatography and mass spectrometry (GC/MS). Other CLs are the more polar lipids such as aldehydes, alcohols, ketones, wax esters or non-volatile fatty acid derivatives (NFADs) some of which are not detected by standard GC/MS methods without prior derivatisation (Buckner, 1993; Kühbandner & Ruther, 2015). As a result, they are often neglected in studies investigating mate recognition in insects and the term CHCs is often erroneously used interchangeably with CLs. To unambiguously show the behavioural effect of CHCs in recognition processes it is however necessary to separate the CHCs from the more polar CLs prior to their use in behavioural bioassays. This can be achieved easily by fractionating complete CL extracts on a SiOH column. In addition, recent studies have shown that more polar lipids can similarly elicit behavioural responses in insects and are likely used as contact sex pheromones far more often than previously thought (Yasui et al., 2003; Eliyahu et al., 2008; Kühbandner et al., 2012; Salerno et al., 2012; Stökl et al., 2014; Keppner et al., 2017).

In *Nv*, males recognise females based on the females' CHCs alone and do not rely on additional more polar messengers (Steiner et al., 2006). The CHC profile in *Nasonia* consists

of hydrocarbons ranging from C25 to C37 including n-alkanes, mono-, di-, tri- and tetramethylalkanes as well as few alkenes (Carlson et al. 1999; Steiner et al. 2006; Niehuis et al. 2010; Buellesbach et al. 2013, 2018; Mair et al. 2017). Both quantitative and qualitative differences in the composition of CHCs exist between females and males of *Nv* (Carlson et al., 1999; Steiner et al., 2006; Buellesbach et al., 2013, 2018). Compared to males, *Nv* females possess higher relative amounts of hydrocarbons with chain lengths shorter than C30 as well as higher relative amounts of methyl-branched alkanes with central branching positions (e.g. 9-, 11-, 13-, 15-methylalkanes or 9,x-, 11,x-, 13,x-, 15,x-dimethylalkanes) whereas males possess higher relative amounts of methyl-branched alkanes with marginal branching positions (e.g. 3-, 5-, 7-methylalkanes or 3,x-, 5,x-, 7,x-dimethylalkanes) as well as higher relative amounts of alkenes (Steiner et al., 2006; Buellesbach et al., 2013, 2018). Males of *Nv* use the sex-specific differences in the CHCs to distinguish females from males by means of antennal contact during encounters (Steiner et al., 2006). In bioassays, female CHCs applied to dummies (solvent-washed male corpses) elicit arrestment and courtship behaviour including copulation attempts, whereas male CHCs do not (Steiner et al., 2006; Mair et al., 2017).

In addition to sex-specific differences, the composition of CLs differs among all four *Nasonia* species (Carlson et al., 1999; Steiner et al., 2006; Raychoudhury, Desjardins, et al., 2010; Buellesbach et al., 2013; Mair et al., 2017). More specifically, compared to females of *Ng*, females of *Nv* possess higher relative amounts of n-alkanes and monomethylalkanes, whereas females of *Ng* possess higher relative amounts of di-, tri- and tetramethylalkanes (Mair et al., 2017). In addition, Niehuis et al. (2010) showed that males of *Nv* possess larger relative amounts of the three alkenes 9-C31ene, 9-C33ene and 7-C33ene than males of *Ng*. However, in bioassays with fractionated female extracts (containing only CHCs), *Nv* males showed courtship and copulation attempts equally often towards both dummies applied with CHCs of *Nv* females and those applied with CHCs of *Ng* females (Mair et al., 2017). Furthermore, in bioassays with living couples, *Nv* males seem to even court females of *T. sarcophagae*, a species closely related to the genus *Nasonia* which possesses a relatively similar CHC composition compared to *Nv* (Niehuis et al., 2013; Buellesbach et al., 2018). Overall, *Nv* males are hardly selective in the choice of their mating partners (Giesbers et al., 2013; Buellesbach et al., 2014, 2018) indicating that mating with the wrong partner does not impose considerable fitness costs on them. Although repeated courtship and mating reduce male longevity (Burton-Chellew, Sykes, et al., 2007), this has probably only little effect on overall male fitness in nature considering that males with continuous contact to females lived

for more than nine days in bioassays, a time span in which most or all females have typically emerged from the host puparium and mated already (Mair & Ruther, 2018). In addition, males can mate multiple times before suffering from sperm depletion (seven or more matings; Whiting 1967; Ruther et al. 2009; Chirault et al. 2016). Apart from losing time and energy spent in misdirected courtship, single mistakes in mate choice are thus not severely costly for *Nv* males.

Similar to *Nv*, males of the other three *Nasonia* species also engage in courtship and copulation with heterospecific females (Giesbers et al., 2013; Buellesbach et al., 2014; Mair et al., 2017). Solely in *Ng* it has been shown that males show significant discrimination against heterospecific mating partners in living couples: They refrain from starting courtship more often when confronted with females of *No* than with conspecific females (Buellesbach et al., 2014) and start courtship faster when confronted with conspecific as compared to *Nv* females (Mair et al., 2017). This preference for conspecific females was absent, however, in bioassays with dead females and when males were confronted with female extracts (CLs) or fractionated female extracts (CHCs) applied to dummies (Giesbers et al., 2013; Mair et al., 2017). This indicates that *Ng* males use additional species-specific characteristics such as visual cues, tactile cues or differences in the females' behaviour to differentiate between con- and heterospecific mating partners. Furthermore, the class of substances used by males in the recognition of females differs between *Nv* and *Ng*. While males of *Nv* rely solely on the females' CHCs (Steiner et al., 2006; Mair et al., 2017), conspecific courtship in *Ng* males is only induced when confronted with complete CL extracts of conspecific females including the more polar lipids (Mair et al., 2017). Surprisingly, although conspecific female CHCs are not sufficient to induce courtship in *Ng* males, heterospecific female CHCs are (Mair et al., 2017). A shift to other chemical messengers used in mate recognition must therefore have happened in *Ng* (Mair et al., 2017). However, the cause leading to this shift and the reasons why heterospecific female CHCs remain attractive to *Ng* males are still unclear..

In *No*, bioassays with complete CL extracts indicate that males of *No* might be able to discriminate against heterospecific females belonging to any other *Nasonia* species (Giesbers et al., 2013; Buellesbach et al., 2013). In mating trials with living couples, however, males of *No* courted con- and heterospecific females equally often (Buellesbach et al., 2014). Nevertheless, a more detailed comparative study of the different males' discriminative abilities which, instead of merely looking at presence/absence of courtship, includes more subtle behavioural parameters such as temporal behavioural patterns is widely lacking to date. In addition, the chemical basis of mate recognition in *No* and *Nl* has not been

solved in detail yet and the potential effects that male discriminatory behaviour has in more natural environments under microsympatry still needs to be elucidated. Based on the evidence gained so far, it is however unlikely that males of *Nasonia* contribute much to interspecific prezygotic reproductive isolation among the *Nasonia* species.

(3) COURTSHIP AND FEMALE MATE DISCRIMINATION – THE MALE ORAL SEX PHEROMONE

In contrast to males, females of all *Nasonia* species discriminate against heterospecific males (Raychoudhury, Desjardins, et al., 2010; Giesbers et al., 2013; Buellesbach et al., 2014; Ruther et al., 2014; Mair et al., 2017, 2018). One means by which a female gets the possibility to choose between different mating partners is the male's courtship display. During courtship, the male mounts the female and starts moving its head along the female's antennae, a behaviour termed head-nodding. Head-nodding comes in repetitive series consisting of species-specific patterns of long and short intervals between nodding movements, and is accompanied by stroking movements of the male's antennae and legs over the female's head and eyes (Barrass, 1960, 1961; van den Assem, Jachmann, et al., 1980; van den Assem & Werren, 1994; Jachmann & van den Assem, 1996; van den Assem & Beukeboom, 2004). Head-nodding cycles typically consist of one slow upward stroke followed by several faster nods, a temporal pattern which is likewise exhibited in all *Nasonia* species but differs in details such as the number of fast nods and the length and number of head-nodding cycles (van den Assem, Jachmann, et al., 1980; van den Assem et al., 1981; Ruther et al., 2010; Ruther & Hammerl, 2014). Along with these courtship movements, an oral male sex pheromone is transferred from the male's mouthparts to the female's antennae (van den Assem, Jachmann, et al., 1980; van den Assem et al., 1981; Ruther et al., 2010; Ruther & Hammerl, 2014) potentially giving the female another means to discriminate between different mating partners. When accepting the courting male, the female shows receptivity by flattening the antennae, lowering the head and opening the genital orifice, and copulation follows (van den Assem & Vernel, 1979; van den Assem, Jachmann, et al., 1980; van den Assem, 1986). After copulation, the male usually returns to the courtship position and performs several more head-nodding movements before unmounting (van den Assem & Visser, 1976). It is likely that during this post-copulatory courtship the male transfers an additional amount of the oral sex pheromone. As a result of courtship, receptivity and

copulation, a switch happens in the females' behaviour: Mated females are no longer attracted to the male abdominal sex pheromone (pheromone switch; van den Assem 1986; Ruther et al. 2007, 2014; Steiner and Ruther 2009; Ruther and Hammerl 2014; Lenschow et al. 2018), typically refrain from mating again (receptivity switch; Holmes 1974; van den Assem and Visser 1976; Grillenberger et al. 2008) and become restless instead and switch to dispersal and host-seeking behaviour (King, 1993; King et al., 2000; Steiner & Ruther, 2009a; Ruther et al., 2014).

The transfer of the oral male sex pheromone during courtship is a prerequisite for the induction of receptivity in females. During head-nodding the male extrudes its mouthparts and transfers the aphrodisiac to the female's antennae (van den Assem, Jachmann, et al., 1980; van den Assem et al., 1981; Ruther et al., 2010; Ruther & Hammerl, 2014). By experimentally covering the mouthparts with a drop of glue, the transfer of the pheromone can be inhibited resulting in the prevention of female receptivity signalling (van den Assem, Jachmann, et al., 1980; Ruther et al., 2010). The substances that are responsible for inducing receptivity are however unknown to date (Ruther & Hammerl, 2014) and the establishment of experimental procedures allowing to test pheromonal extracts, fractions of these extracts and synthetic pheromone components has turned out to be rather difficult (personal observation, both authors). All attempts eliciting receptivity in females in the absence of an unconstrained courting male failed preventing a bioassay-guided approach for pheromone identification so far. It seems that the behavioural pattern and the exact timing of the application of the pheromone is crucial (personal observation, both authors). Van den Assem et al. (1980) suggested the pheromone to be volatile. They puffed the headspace of courting couples into a chamber containing a constrained couple with a sealed male and reported that the female became receptive. Our frequent attempts to repeat this experiment, however, were unsuccessful so far. Sealing the male prevented the female from signalling receptiveness, but we were never able to reverse this effect by applying the headspace of courting couples. We therefore suggest that the ominous aphrodisiac of *Nv* males is non-volatile. Females of *Nv*, *Ng* and *Nl* typically show receptivity during the first upwards stroke in a head-nodding series (van den Assem & Werren, 1994). It is thus likely that the pheromone has to be applied in due time with this movement. In addition, whether the oral sex pheromone is also involved in female discrimination against heterospecific males necessitates further investigation.

After mating, *Nv* females are no longer attracted to the male abdominal sex pheromone. This pheromone switch occurs fast (within minutes) and is long-lasting (at least six days; van den Assem 1986; Ruther et al. 2007, 2014; Steiner and Ruther 2009b; Ruther and

Hammerl 2014). As during copulation a single male usually transfers sufficient sperm to fertilise all eggs the female is likely able to lay (Holmes, 1974; Chirault et al., 2016), not being attracted to male territorial markings is reasonable for mated females and gives them the opportunity to switch to host-seeking behaviour instead (Ruther et al., 2007; Grillenberger et al., 2008). In addition, Lenschow et al. (2018) argue that the pheromone switch has evolved as a result of a male strategy to prevent females from encountering and copulating with other males. Responsible for the pheromone switch is the application of the male oral sex pheromone to the females antennae during courtship (Ruther et al., 2010). Courtship movements, copulation, the transfer of sperm or ejaculate and post-copulatory courtship, on the other hand, are not necessary (Ruther et al., 2010; Ruther & Hammerl, 2014). In bioassays, the pheromone switch can even be triggered by merely bringing the antennae of virgin females into contact with male-derived head extracts (Ruther & Hammerl, 2014). More precisely, the active components of the oral sex pheromone that elicit the pheromone switch have been identified as the three fatty acid esters ethyl oleate, ethyl linoleate and ethyl α-linolenate (Ruther & Hammerl, 2014). The mere antennal contact with these three substances thus resulted in a change in the females' response to the abdominal sex pheromone. A study on the neuromodulatory mechanisms underlying this behavioural plasticity showed that it involves the release of dopamine (DA; Lenschow et al. 2018). In bioassays, feeding the DA receptor antagonist chlorpromazine prevented the pheormone switch while the injection of DA into virgin females rendered them unresponsive to the abdominal sex pheromone. As dopamine is also involved in appetitive olfactory learning (Waddell 2013; Lenschow et al. 2018), this suggests that DA plays a key role in mediating olfactory plasticity in *Nv*. The pheromone switch in *Nv* females is not only induced by conspecific males but can be similarly induced by heterospecific males and a similar switch has been demonstrated in females of *Ng* (Ruther et al., 2014). It is thus likely that the pheromone switch and the substances eliciting the switch are not species-specific but instead represent an ancestral state in the *Nasonia* genus.

Another behavioural switch that happens in females after mating concerns the females' willingness to re-mate. Females of *Nv* typically mate only once during their lifetime (Holmes, 1974; van den Assem & Visser, 1976; Grillenberger et al., 2008). When being courted by a second male after having already mated previously, females usually refuse to become receptive and re-mating does not occur (Holmes, 1974; van den Assem & Visser, 1976). In contrast to the pheromone switch, however, this receptivity switch is connected to the post-copulatory courtship exhibited by males after copulation (van den Assem & Visser, 1976;

Boulton & Shuker, 2015). The actual copulatory act and the transfer of seminal fluids, on the other hand, are not important. Van den Assem and Visser (1976) observed in behavioural bioassays, that females showed a second receptivity signal during post-copulatory courtship. They thus hypothesised that females need to show receptivity twice before the receptivity switch happens. As courtship involves the transfer of a pheromone, it is likely that this is also true for post-copulatory courtship. The male oral sex pheromone consists of various compounds that are not involved in the pheromone switch (Ruther & Hammerl, 2014). It is thus likely that some of these compounds are involved in the receptivity switch, a hypothesis which still necessitates further investigation. Reported re-mating rates differ among *Nasonia* species and between different *Nasonia* strains (Leonard & Boake, 2008; Geuverink et al., 2009). In addition, re-mating appears to increase in strains having been reared in the laboratory over prolonged time (van den Assem & Jachmann, 1999; Burton-Chellew, Beukeboom, et al., 2007). Studies investigating the receptivity switch therefore need to take into account the individual history of the investigated strains and should ideally work with field-collected outbred strains.

(4) OLFACTORY HOST FINDING – THE ROLE OF HOST HABITAT ODOURS AND HOST KAIROMONES

After mating, females of *Nv* become restless, are more ambitious of flying and start searching for hosts (King, 1993; King et al., 2000; Ruther et al., 2014). Because hosts are usually distributed patchily in the environment occurring in birds' nests and on rotting carcasses, finding adequate hosts is a challenging task for females. Grillenberger et al. (2008) found a clear isolation between populations of *Nv* at two sampling sites in the Netherlands and Germany located about 300 km apart. Nevertheless, *Nv* females are able to disperse over long distances as indicated by the lack of genetic population substructure over a range of 100 km in a field study in the US (Grillenberger, Gadau, et al., 2009). During long-distance dispersal, females are supposedly drifting as aerial plankton by the use of wind currents to reach new habitats (Grillenberger, Gadau, et al., 2009). For orientation over intermediate and short distances on the other hand, active chemotaxis towards host habitat odours has been suggested (Whiting, 1967). Consistently, material taken from birds' nests and carcasses attracts mated *Nv* females (Peters, 2011; Frederickx et al., 2013). One compound which has been shown to be particularly attractive to mated females is dimethyldisulphide (DMDS), a

major component found in the headspace of decaying meat (Kasper et al., 2012). Mated females tested in olfactometer experiments showed a clear affinity towards DMDS whereas all other tested compounds of rotten meat extracts did not elicit preferential behaviour (Frederickx et al., 2013). By using host habitat related chemical cues, adult *Nv* arrive at carcasses at an early stage of decomposition when adult host flies and larvae are already present, but host pupae have not developed yet (Voss et al., 2009). After arriving at a suitable host habitat, females most likely rely on short-range or contact kairomones originating from host fly puparia to find and identify adequate hosts on the carcass or inside the birds' nests, respectively. In bioassays using a still-air olfactometer, mated females preferred the odor of hosts over that of conspecific males right after mating (Steiner & Ruther, 2009a). During the inspection of the fly puparium, females are able to distinguish between different fly species and are even able to assess the parasitisation status of the encountered host (King & Rafai, 1970; Rivers & Denlinger, 1995a).

Female dispersal and the chemical basis of host location in the other *Nasonia* species have not been investigated to date. It is, however, likely that all *Nasonia* females use similar modes of transport and chemotaxis. Whether they respond to the same chemical compounds during their search for new host patches, however, needs further investigation. As *Ng* is specialised on pupae of *Protocalliphora*, it is likely that females of *Ng* (and likely also females of *No*) are not attracted to odours originating from carcasses but rely solely on cues associated with birds' nests.

(5) OVIPOSITION AND SEX RATIO ADJUSTMENT – CHEMICAL ASSESSMENT OF HOST QUALITY

When females encounter a host puparium after arrival at a new host patch, they need to decide firstly whether to lay eggs, secondly how many eggs to lay and thirdly how many eggs shall develop into females or males, respectively. *Nasonia* wasps, like all hymenopterans, are haplodiploid, i.e. females are diploid and emerge from fertilised eggs, whereas males are haploid and develop from unfertilised eggs (Heimpel & de Boer, 2008). During oviposition, mated females are capable of actively adjusting the sex ratio of their offspring in response to varying environmental factors essential for offspring survival and performance such as the number, quality and parasitisation status of the hosts (Verhulst et al., 2010).

A high nutritional value of host pupae positively influences offspring fitness which is reflected in a shorter development time and increased body size of the adults (Rivers & Denlinger, 1995a; Hoedjes et al., 2014). In contrast, females emerging from hosts of lower quality are smaller and produce fewer eggs. Despite compensation through host feeding in the course of oviposition, lipids acquired prior to emergence are essential and limiting for adult females (Rivero & West, 2002, 2005; Sykes et al., 2008). In addition, males having developed in hosts rich in linoleic acid produce higher amounts of HDL and sperm, and are thus most likely able to attract and inseminate more females (Blaul & Ruther, 2011). The amount of host nutrients available per individual, however, decreases with increasing parasitoid clutch size or when a host is parasitised by more than one female. In addition, due to local mate competition (LMC) between male offspring at the natal host patch after emergence, females typically produce increased numbers of males under superparasitism to increase the mating success of their male offspring in the competition for mates (Werren, 1980, 1983; Shuker et al., 2006; Burton-Chellew et al., 2008). Therefore, it is crucial for the females' fitness to optimise clutch size and offspring sex ratio after assessing host quality and parasitisation status.

When encountering a host, the female inspects the fly puparium from outside with her antennae and the tip of her abdomen before drilling into it with her ovipositor (Edwards, 1954; King & Rafai, 1970). The tip of the ovipositor possesses pores containing chemoreceptors which are likely used to chemically inspect the status of the host (Edwards, 1954; King & Rafai, 1970). If the host is deemed suitable for oviposition, venom is injected into the fly pupa and eggs are laid onto the pupa inside the puparium (Wylie, 1965; Ratcliffe & King, 1967). During the process of drilling, females are able to assess the nutritional quality as well as the pre-parasitisation status of the host pupa (Wylie, 1965; King & Rafai, 1970; Blaul & Ruther, 2011).

Nv females discriminate between pupae of different fly species and prefer pupae of *Sarcophaga* spp. over those of *Musca domestica* (Rivers & Denlinger, 1995a). In addition, they discriminate between different host sizes and hosts with different nutritional values (Rivers & Denlinger, 1995a; Blaul & Ruther, 2011). On dead or small hosts, less eggs are laid and offspring sex ratios are shifted in favour of males (Rivers & Denlinger, 1995a). In addition, females prefer LA-enriched hosts over those poor in LA (Blaul & Ruther, 2011). Given the essential function of LA in the production of sperm and HDL, this is highly adaptive. Additional cues used for the assessment of host quality are, however, to be discovered and offer promising opportunities for further research.

In addition to general host quality, *Nv* females are able to discriminate between parasitised and non-parasitised hosts (Wylie, 1965; King & Rafai, 1970; Holmes, 1972; Werren, 1980; King & Skinner, 1991; Shuker & West, 2004) and are even able to distinguish hosts pre-parasitised by conspecific females from those previously parasitised by heterospecific ones (Ivens et al., 2009). In general, pre-parasitised hosts are rejected more often (Ivens et al., 2009). If females decide to oviposit nonetheless, fewer offspring and higher relative numbers of males are produced (Wylie, 1965; Holmes, 1972). The means by which ovipositing females assess the parasitisation status of hosts has not been identified yet, but chemical messengers are likely involved (Wylie, 1965; King & Rafai, 1970; Holmes, 1972). Holmes (1972) hypothesised that female venom injected into the fly pupa during oviposition might act as a pheromonal signal and King and Rafai (1970) recognised changes in the hosts' haemolymph composition after the injection of female venom.

The venom of *Nv* is acidic and consists of different amines, peptides and non-glycosylated proteins of mostly mid to high molecular weight (Rivers et al., 2006). In total, 79 proteins and peptides have been identified. They belong to different functional groups including proteases, peptidases, protease inhibitors, three enzymes with functions in carbohydrate metabolism (chitinase, trehalase and glucose dehydrogenase), DNA metabolism (apyrases, endonucleases) and glutathione metabolism (γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase), esterases (e. g. acid phosphatase and arylsulphatase), recognition/ binding proteins (e. g. a chitin-binding-protein and odorant-binding proteins) and immune related proteins (calreticulin, immunoglobulin-like protein; de Graaf et al. 2010; effects of individual compounds are discussed in detail in Danneels et al. 2010). The most obvious effect of *Nv* venom on host pupae is the developmental arrestment followed by the death of the pupae after some time depending on both the fly species and pupal age (Rivers & Denlinger, 1994a). Five to ten minutes after envenomation a blackening of the injection site can be observed, a result of melanisation of the damaged tissue initiated by the host as a defense mechanism (Rivers et al., 1993). Instead of provoking paralysis or muscular contractions, a cascade of metabolic changes is initialised in the host leading to the mobilisation of nutrients for the developing wasp larvae (Rivers et al., 1993). Host immune responses on the other hand are impaired, oxygen consumption drops sharply and concentrations of oxaloacetate, trehalose, glycogen and haemolymph amino acids increase (Rivers & Denlinger, 1994b; Rivers, Ruggiero, et al., 2002). At the cellular level, plasma membrane permeability changes and increased influx of Na^{+} leads to cellular swelling and, finally, cell death (Rivers, Rocco, et al., 2002). In addition, $[Ca^{+2}]_i$ is mobilised from

intracellular storage, cytosolic $[Ca^{+2}]_i$ levels rise, phospholipase A_2 is activated and, as a consequence, fatty acid synthesis is stimulated (Rivers, Rocco, et al., 2002). Three to four days after envenomation of *S. bullata*, pyruvate concentrations decrease and elevated levels of lipid are found in the host's fat body and haemolymph, a change which coincides with the last larval molt and intensified feeding activity of the developing parasitoid (Rivers & Denlinger, 1994b, 1995b; Rivers & Yoder, 1996). These tremendous changes in the composition and relative amount of different substances found in hosts after envenomation have notable potential of being used in host assessment by female wasps. Which substances are of relevance during host assessment and whether the used substances are constituents of the venom or rather emerge by means of physiological changes induced by venom injection remains to be elucidated, however. Further investigations of the chemoreceptors found on the ovipositor as well as controlled bioassays might be promising.

Females of *Ng* are also able to discriminate between different host species and prefer pupae of *Protocalliphora sialia* over those of *Sarcophaga bullata* (Desjardins et al., 2010) which is in accordance with the occurrence of *Ng* in birds' nests rather than on carcasses. Bioassays on host discrimination in *Nl* and *No* have not been conducted so far, but it is likely that they are similarly able to distinguish different host species and host qualities. Also, taking a deeper look into the venom composition of the other three *Nasonia* species might give insights into mechanisms used by females to further differentiate between con- and heterospecifically pre-parasitised hosts.

ASSOCIATIVE OLFACTORY LEARNING

The olfactory localisation of hosts by *Nasonia* females is not completely inherent but employs a dynamic learning and conditioning scheme which depends on the memory of host-associated chemical cues. Females of *Nv* are able to memorise odours they perceive during host assessment and oviposition and use these cues during the subsequent search for new hosts. The inspection of a single host puparium for one hour in the presence of the synthetic volatile furfuryl heptanoate (FFH; the conditioned stimulus) for example led to the preference of FFH in subsequent olfactometer bioassays (Schurmann et al., 2009). This conditioning however was non-permanent and the preference vanished after four days. Nevertheless, Schurmann et al. (2012) were able to increase memory effects to six days by elongating the duration of inspection, repeating conditioning procedures and allowing the

females to perform host-feeding or oviposition in the presence of the conditioned stimulus (cinnamon odour). The physiological mechanisms leading to the formation of a long-term memory are dependent on protein synthesis which is indicated by a decreased memory retention to a maximum of three days after the injection of the transcription inhibitor actinomycin D after training (Schurmann et al., 2012).

The neuromodulatory mechanisms underlying associative learning in insects involve a variety of different chemical messengers which interact at different levels of the olfactory system (Menzel & Müller, 1996). Two neuromodulators found to be of specific importance in learning are the two biogenic amines dopamine (DA) and octopamine (OA; Unoki et al. 2005, 2006). While DA has usually been associated with aversive learning (i.e., learning to avoid a specific behaviour by pairing it with an unpleasant stimulus) in insects, OA has predominantly been associated with appetitive learning (i.e., learning to intensify a specific behaviour by pairing it with a pleasant stimulus; Schwaerzel et al. 2003; Unoki et al. 2005). However, although OA seems to be sufficient in inducing appetitive learning in some insects (e.g. honeybees and crickets; Hammer and Menzel 1998; Matsumoto et al. 2015), it has been shown lately that appetitive learning involves both DA and OA in others (e.g. *Drosophila melanogaster*; Burke et al. 2012). Similar to *D. melanogaster*, a recent study indicates that both DA and OA are also important for appetitive learning in *Nv* (Lenschow et al., 2018). In behavioural bioassays using oviposition as a reward, mated females of *Nv* have been readily conditioned to being attracted again to the male abdominal sex pheromone, thus reversing the pheromone switch. However, after having been fed DA or OA receptor antagonists prior to conditioning, appetitive learning was prevented. In addition, the oviposition reward could be mimicked completely by the injection of DA and partially by the injection of OA into the abdomen of mated females prior to exposure to the male sex pheromone. This indicates that, similar to *D. melanogaster*, DA and OA act in a concerted manner on different levels in the olfactory system during appetitive learning in *Nv* (Burke et al., 2012; Lenschow et al., 2018).

Memory retention after associative olfactory conditioning differs among *Nasonia* species (Hoedjes et al., 2012; Hoedjes & Smid, 2014). In experiments involving only one single conditioning event, memory lasted up to five days in *Nv* and *Nl*, whereas in *Ng* the effect already vanished after two days (Hoedjes et al., 2012). In addition, in contrast to females of *Nv,* females of *Ng* depended on a second conditioning treatment to form long-term memory (Hoedjes & Smid, 2014). One possible reason why these differences in memory retention have evolved is the difference in host preference among the *Nasonia* species (Hoedjes et al., 2011, 2012). *Nv* and *Nl* are generalists which accept pupae of a wide

variety of different fly species as hosts (Darling & Werren, 1990; Desjardins et al., 2010). Different fly species prefer different habitats such as birds' nests (e.g. bird blowflies, *Protocalliphora* spp.; Bennett and Whitworth 1992) or carcasses (e.g. several species of Sarcophagidae (flesh flies) and Calliphoridae (blow flies); Denno and Cothran 1976) for oviposition, and the availability of fly pupae in different habitats in the field likely changes over time (Villet et al., 2017). A dynamic learning scheme which allows females to focus during the search for hosts on odours connected to recent successful oviposition experiences might thus be particularly adaptive in these two species (Schurmann et al., 2009, 2012; Hoedjes et al., 2011, 2012). In contrast, *Ng* is primarily specialised on pupae of *Protocalliphora* spp. which are obligate parasites of birds (Darling & Werren, 1990). Considering the consistency of relying solely on birds' nest odours, depending on stronger innate preferences during host-seeking might therefore be advantageous for them (Hoedjes et al., 2011, 2012).

OLFACTORY PERCEPTION – ANTENNAL MORPHOLOGY, ODORANT-BINDING PROTEINS AND CHEMOSENSORY RECEPTORS

The body parts most obviously used in chemoreception in *Nasonia* are the wasps' antennae, but chemosensory sensillae have also been found on the wasps' tarsal claws, maxillary palps, labial palps and on the female's ovipositor (Slifer, 1969). Three types of chemosensory sensillae have been identified in *Nv* (Slifer, 1969; Wibel et al., 1984): Thick-walled sensillae are bristle-shaped hollow chitinous structures which contain a single pore on the sensillar tip where the dendrites are exposed. Thick-walled sensillae are mainly located on the antennal tip (11^{th} and 12^{th} antennal segments) as well as on tarsal claws, maxillary and labial palps. In contrast, thin-walled sensillae and multiporous plate sensillae are perforated by many small pores. While thin-walled sensillae are bristle-shaped, plate organs are prominent elongated structures connected tightly to the cuticular surface. Both thin-walled sensillae and multiporous plate sensillae are located on segments three to eleven, sparing the antennal tip (Slifer, 1969). The abundance of the different types of sensillae are sex-specific, with females carrying more multiporous plate sensillae and thick-walled sensillae than males (Slifer, 1969; Wibel et al., 1984).

Insect sensillae are filled with sensillar lymph, an aqueous liquid which contains various proteins such as odorant-binding proteins (OBPs) and chemosensory proteins (CSPs), i.e.

small water-soluble proteins which help to transport the often hydrophobic odour molecules through the aqueous liquid (Vieira & Rozas, 2011; Vieira et al., 2012; Pelosi et al., 2014). During olfaction, odour molecules enter the sensillum through the sensillar pores, bind to OBPs or CSPs and diffuse to chemosensory receptors which are located in the dendritic membrane of chemosensory neurons reaching into the sensillar lymph (Vieira & Rozas, 2011). The chemosensory receptors interact with the odour molecules, and the presence of this interaction is subsequently communicated via electrical signals to the nervous system where the information is processed (antennal lobe, mushroom bodies, lateral horn). Chemosensory receptors are commonly classified into two main groups: odorant receptors (ORs) associated with the perception of food odours and pheromones and gustatory receptors (GRs) which include among others highly conserved carbon dioxide receptors (Jones et al., 2007; Kaupp, 2010).

The number of gene loci coding for OBPs, CPSs and chemosensory receptors are often discussed as indicators of the complexity of olfactory behaviour in animals (Robertson & Wanner, 2006; Robertson et al., 2010). In the genome of *Nv*, 10 predicted CSPs and 90 predicted OBPs have been identified, 59 of which have full expressed sequence tags (EST) support indicating that at least two thirds of the OBP sequences are also expressed (Werren, Richards, Desjardins, Niehuis, Gadau, et al., 2010; Vieira et al., 2012; Pelosi et al., 2014). All 90 OBPs of *Nv* have orthologues in *Nl* and *Ng* (Vieira et al., 2012). Compared to the 21 OBPs and 6 CSPs in the honey bee *Apis mellifera* and the 52 OBPs and 4 CSPs in *Drosophila melanogaster* (Sánchez-Gracia et al., 2009; Pelosi et al., 2014), the genus *Nasonia* thus seems to possess an exceptionally large OBP and CSP gene family. In accordance with the high number of OBPs and CSPs, the number of loci coding for ORs in *Nv* is also notably large compared to other insects (225 as compared to 170 in *A. mellifera* and 62 in *D. melanogaster*; Robertson and Wanner 2006; Robertson et al. 2010). In accordance with the *Nasonia* lifestyle, although possessing 58 predicted GRs, no predicted carbon dioxide receptors have been found in *Nv* (Robertson et al., 2010).

The *Nasonia* OBPs, ORs and GRs have not yet been functionally characterised. Neither the ORs detecting the different pheromone components nor the olfactory sensillae housing the pheromone sensing olfactory receptor neurons have been identified so far. The neurophysiological mechanisms underlying the stereospecific perception of chiral semiochemicals by insects are poorly understood. A comparative study of the *Nasonia* olfactory system offers great opportunities to achieve major advances, because the species specificity of the chemical information is encoded by the chiral stereoisomers RS and RR

which are perceived differentially by *Nv* and the other *Nasonia* species, respectively (Niehuis et al., 2013). The availability of whole genome sequences and annotated olfactory gene families for the *Nasonia* species will facilitate the application of molecular tools to unravel the molecular mechanisms underlying the perception of a newly evolved chiral pheromone component. Hence, the *Nasonia* model system has the potential to enable important inferences on the molecular basis of enantioselective pheromone perception in insects.

OUTLOOK

Nasonia is an excellent model system for the study of the chemical ecology of hymenopteran parasitoids. The chemical communication system of *Nv* is one of the best understood in insects. Together with easy rearing and handling techniques and the well-established bioassays and experimental procedures this offers fruitful opportunities to broaden the field of *Nasonia* research to get new insights into pheromone evolution and odour perception in insects in general.

Females of *Nasonia* react enantio-selectively to the male abdominal sex pheromone components RS and RR. The characterisation of the ORs that are connected to the perception of RS and RR could thus help getting a deeper understanding of the mechanisms involved in enantio-selective odour perception in general. Furthermore, it is still unclear, how the male abdominal sex pheromone of *Nasonia* has evolved and how the evolution was driven by selection pressures imposed by co-occurring species. This question could be addressed, first, by a comparative approach including parasitic wasp species belonging to other pteromalid genera (e.g. *Urolepis*) and, second, by investigating in more detail the differences and similarities in the pheromone communication and behaviour of species which occur in (micro)sympatry with *Nv* (e.g. species of *Muscidifurax*, *Spalangia* or *Pachycrepoides*; Rueda and Axtell 1985; Rueda et al. 1997). Finally, the robustness of the olfactometer bioassay with *Nv* females makes *Nv* a potential model system of great value for the study of the effects of anthropogenic substances on the chemical communication of insects (see for example Tappert et al. 2017).

3. Territoriality and behavioural strategies at the natal host patch differ in two microsympatric *Nasonia* species

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ABSTRACT

Territoriality occurs in a wide variety of animal taxa. The defence of valuable resources, which in the case of territoriality are bound to a specific location, gives the defender priority of access to these resources. Males often defend areas in which the chance to meet females is high and territoriality frequently includes pheromonal marking. When closely related species co-occur within the same environment, different behavioural strategies frequently evolve to avoid reproductive interference. Males of the gregarious parasitoid wasp *Nasonia vitripennis* exhibit territorial behaviour on the host from which they emerge. However, descriptions of territorial behaviour have been for the most part anecdotal and quantitative standardised observations in an experimental set-up are lacking. In addition, studies of the behaviour of the other *Nasonia* species that frequently occur in microsympatry, that is, within the same host individual, have rarely been conducted. We investigated and compared territoriality in two species of *Nasonia* by extensive video recording of emerging wasps in a microcosm approach. We show that males of *N. vitripennis* meet the concept of territoriality whereas males of *Nasonia girault*i do not. Although *N. girault*i females are already mated when emerging from the host and males do not show territoriality, *N. girault*i males marked the substrate with their abdominal sex pheromone as often as males of *N. vitripennis*. For *N. vitripennis* we further show that, although larger males were more often territorial, experience of being in the territorial position was particularly important for winning territoriality contests. Finally, we investigated differences in the pattern of emergence and dispersal between the two species and discuss how the different behavioural strategies may help them avoid reproductive interference.

INTRODUCTION

Many animals defend limited resources such as food, oviposition sites or mating partners against their competitors. This occurs in more, or less, aggressive contests in which one individual typically gains dominance while the other exhibits subordinate behaviour. In social groups, repeated contests frequently result in relatively stable dominance–subordinance relationships between pairs of individuals and complex dominance hierarchies may develop (Kaufmann, 1983). The defence of resources that are bound to specific locations, for example oviposition sites, specific food patches, adequate breeding sites or locations frequently visited by females, is termed territoriality. The successful holder of a territory gains sole or priority of access to the resources that are present (Kaufmann, 1983; Maher & Lott, 1995). Defence of a resource, however, does not necessarily imply aggressive interactions. The behaviours involved range from direct aggression to complex behavioural displays to mere advertisement by visual presence, acoustic signalling or scent marking (Baker, 1983). In males, chemical messengers used to scent mark the territory can function as attractants for females and indirect indicators of male quality (Johansson & Jones, 2007).

Territoriality exhibited by males at locations where encounters with females are likely to occur have been observed in a wide variety of animal taxa ranging from mammals (Clutton-Brock, 1989) and other vertebrates (Cuadrado, 2006; Eriksson & Wallin, 1986; Roithmair, 1994; Spence & Smith, 2005) to various arthropods (Christy, 1987; Edwards & Dimock, 1991; Fitzpatrick & Wellington, 1983; Suter & Keiley, 1984). In insects, males establish territories near or at oviposition sites, on routes to oviposition sites, near or at females' foraging sites, near or at nest entrances from which virgin females might emerge or directly at female pupae which are guarded until adult females eclose (Fitzpatrick & Wellington, 1983).

Territoriality at female emergence sites has also been described in parasitoid wasps, for example the ichneumonid wasp *Lytarmes maculipennis* (Godfray, 1994), several species of scelionid wasps (Waage, 1982; Wilson, 1961) and the pteromalid wasp *Nasonia vitripennis* (van den Assem, Gijswijt, et al., 1980). Species of the last two examples are protandrous (i.e. males emerge earlier than females) and gregarious (more than one wasp develops within one host) or quasi-gregarious (only one wasp develops per host, but hosts are clumped). Protandry gives males the opportunity to set up territories before females emerge at the same host patch. Females can then be intercepted, courted and mated before they leave the natal host patch and seek oviposition sites (Godfray, 1994; Wiklund & Fagerström, 1977).

When two species that are reproductively isolated by postzygotic isolation mechanisms occur in sympatry they usually evolve prezygotic strategies to avoid reproductive interference and interspecific mating (Gröning & Hochkirch, 2008; Noor, 1999). In insects, such strategies often include mechanisms connected directly to mate recognition and mate choice, for example differences in courtship behaviour (van den Assem & Werren, 1994; Tomaru & Oguma, 1994) or discrimination between conspecific and heterospecific mating partners by chemical messengers (Singer, 1998; Wyatt, 2014). However, reproductive interference can also be avoided by developing differences in the temporal mating pattern or by shifting mating sites within the same habitat (Hardeland, 1972; Kuno, 1992).

A mating site shift has been suggested in the parasitoid wasp genus *Nasonia* (Drapeau & Werren, 1999; Giesbers et al., 2013; Leonard & Boake, 2006; Ruther et al., 2014). The genus consists of four species which all parasitise pupae of cyclorrhaphous flies (Darling & Werren, 1990; Raychoudhury, Desjardins, et al., 2010; Whiting, 1967) but differ in various aspects of their mating behaviour. These include different degrees of interspecific mate discrimination during courtship as well as differences in the male sex pheromone composition and the mating sites (Buellesbach et al., 2014; Diao et al., 2016; Drapeau & Werren, 1999; Giesbers et al., 2013; Leonard & Boake, 2006; Mair et al., 2017; Niehuis et al., 2013; Ruther et al., 2014). *Nasonia vitripennis* (*Nv*) is cosmopolitan and occurs in sympatry with each of the other three *Nasonia* species: *N. longicornis* (*Nl*) in the western part and *N. giraulti* (*Ng*) and *N. oneida* (*No*) in the eastern part of North America (Darling & Werren, 1990; Raychoudhury, Desjardins, et al., 2010; Raychoudhury, Grillenberger, et al., 2010). All four species are gregarious and females of different species often multiparasitise the same host individual (Grillenberger, van de Zande, et al., 2009). After hatching, larvae feed as ectoparasites on the fly pupa inside the fly puparium, pupate inside the host puparium and emerge after eclosion. As developmental times of *Ng*, *Nl* and *No* are only slightly longer than those of *Nv*, and fly pupae are usually parasitised over 2 or 3 consecutive days, individuals belonging to two different species may emerge simultaneously from the same host puparium (Bertossa et al., 2010). Except for *No* and *Ng*, all four *Nasonia* species are reproductively isolated by postzygotic cytoplasmic incompatibility resulting from infections with different strains of the intracellular bacterium *Wolbachia* (Bordenstein et al., 2001). As a result, no viable hybrids are produced when females consent to heterospecific mating (Breeuwer & Werren, 1990). In mating experiments, females of *Nv* and *No* exhibited strong discrimination against heterospecific males whereas females of *Nl* and *Ng* were less discriminatory (Buellesbach et al., 2014; Giesbers et al., 2013). While females of *Nv* mate

after emergence from the host, almost all females of *Ng* mate inside the host puparium before emergence (Drapeau & Werren, 1999; Giesbers et al., 2013; Leonard & Boake, 2006). This so-called within-host mating in *Ng* has been suggested to have developed as a mechanism to avoid reproductive interference with *Nv* (Drapeau & Werren, 1999).

Although males of all *Nasonia* species produce an abdominal sex pheromone which is highly attractive to virgin females (van den Assem, Jachmann, et al., 1980; Ruther et al., 2007; Steiner & Ruther, 2009a), the composition of this pheromone differs between them. While the pheromone in *Ng*, *Nl* and *No* consists of (4*R*,5*S*)-5-hydroxy-4-decanolide and 4-methylquinazoline, the pheromone of *Nv* contains a third component, the epimer (4*R*,5*R*)-5-hydroxy-4-decanolide which allows females of *Nv* to differentiate between the pheromone of conspecific and heterospecific males (Diao et al., 2016; Niehuis et al., 2013; Ruther et al., 2014, 2007). After emergence, males of *Nv* stay at or near the host, are aggressive towards other males (van den Assem, Gijswijt, et al., 1980; King et al., 1969) and show marking activity which is increased after contact with females (van den Assem, Jachmann, et al., 1980; Barrass, 1969; Steiner & Ruther, 2009b). When *Nv* females emerge, they are courted, and mating follows. After mating, a behavioural switch happens in the females, which results in females no longer being attracted to the male sex pheromone (Ruther et al., 2014, 2007; Ruther et al., 2010). Typically, females of *Nv* mate only once before switching to host-seeking behaviour and multiple mating is rare in nature (Grillenberger et al., 2008; King et al., 2000; Ruther et al., 2014).

The territorial behaviour of *Nv* males after emergence has been described by van den Assem, Gijswijt, et al. (1980) and King et al. (1969), but these descriptions are limited to anecdotal reports rather than quantitative observations in experimental set-ups. Males of *Nv* emerge prior to females (protandry) and the first emerging male usually succeeds in establishing a territory on the host puparium (van den Assem, 1996). The other males of the group have been described as establishing territories in the vicinity of the host, frequently challenging the territorial male on the host, trying to sneak in to gain copulation opportunities when females emerge (van den Assem, 1996; van den Assem, Jachmann, et al., 1980; van den Assem & Vernel, 1979) or wandering off to other hosts from which females are about to emerge (van den Assem, Gijswijt, et al., 1980; King et al., 1969; Shuker et al., 2005). However, no experiments have been conducted to corroborate these anecdotal descriptions by a quantitative analysis of behavioural data gained from a standardised experimental set-up. In addition, few studies have investigated behaviours after emergence in the other three *Nasonia* species. A first approach to comparing species-specific behaviours

of the three *Nasonia* species after emergence in an experimental approach has been conducted by Leonard and Boake (2006). They found a negative relationship between within-host mating/dispersal rate and aggression of males on the host. In the same study, *Nv* and *Nl* showed pronounced aggression on the host and low within-host mating and dispersal rates whereas *Ng* showed no aggression and 100% within-host mating and male dispersal rate. However, more detailed observations of male and female behaviours after emergence are still lacking. Evidence that males of *Ng* leave the natal host patch after emergence raises further questions concerning the production of the abdominal sex pheromone in these males (Niehuis et al., 2013; Ruther et al., 2014). Pheromone biosynthesis is usually costly (Johansson & Jones, 2007; Zahavi, 1975) and in *Nasonia* it involves linoleic acid as a precursor which is also essential for the production of sperm (Blaul & Ruther, 2011; Brandstetter & Ruther, 2016; Wathes et al., 2007). Considering these costs, it is likely that *Ng* males make use of the pheromone in one way or another. Because *Nv* and *Ng* exhibit the most pronounced behavioural differences in the *Nasonia* genus, they represent a good model pair for studying different behavioural strategies occurring in two microsympatric species.

In this study, we investigated the behaviour of *Nv* and *Ng* after emergence from the host in a quantitative microcosm approach by extensive video recording. Filming was conducted in a set-up in which wasps were allowed to emerge freely from a host and given the opportunity to leave the natal host patch to either start ovipositing (females) or seek an alternative behavioural strategy by establishing territoriality on a second parasitised host from which wasps would emerge a few days later (males). We characterise for the first time *Nv* male territoriality on the natal host patch quantitatively and tested hypotheses associated with the general concept of territoriality as well as hypotheses specific to *Nv* arising from earlier observations (van den Assem, 1986, 1996; van den Assem, Gijswijt, et al., 1980; van den Assem, Jachmann, et al., 1980; King et al., 1969; Moynihan & Shuker, 2011; Shuker et al., 2005; Tsai et al., 2014a; b).

One prerequisite for territoriality is the presence of a valuable resource, here access to virgin females. As females of *Nv* emerge from the host as virgins, we consider the host to be the valuable resource worth being defended by males. We hypothesise that *Nv* males would show territoriality on the host and that this would be connected to (1) site fidelity, (2) repeated dominance-subordinance interactions in which territory owners win more often than subordinate males and (3) pheromone marking of the host by territorial males but not by subordinate males. In accordance with previous findings (van den Assem, 1996; van den Assem, Jachmann, et al., 1980; Barrass, 1969; Steiner & Ruther, 2009b), we further

hypothesise that marking activity would increase when females were present. In contrast, we hypothesise that *Ng* males would not be territorial and would less often be involved in dominance–subordinance interactions than *Nv* males. If pheromone marking is shown by *Ng* males, we predicted that it would occur less often than in *Nv* and would not be restricted to specific territorial spots.

If the host from which females are about to emerge is indeed a valuable resource, theory predicts that the vacant territory, after removal of the territory holder, would rapidly be taken over by a competitor (Baker, 1983; Davies, 1978). We tested this prediction with a removal experiment with groups of *Nv* males. Based on the observation by van den Assem (1986) and van den Assem, Gijswijt, et al. (1980), we also tested the hypothesis that replacement of a territorial *Nv* male would be achieved less often in larger than in smaller groups. Territoriality is thought to lead to priority of access to the valuable resource by the holder of the territory (Kaufmann, 1983). We therefore studied the mating success of territorial males and predicted that territorial *Nv* males would be more successful than subordinate ones. Subordinate males of *Nv* have been described as gaining mating opportunities by sneaking in when females show receptivity after courtship by another male (van den Assem, 1996; van den Assem, Gijswijt, et al., 1980; van den Assem & Vernel, 1979). During courtship, the courting male mounts the female, orients himself towards the female's head and starts performing head-nodding behaviour during which a sex pheromone is transferred from the male's mandibular glands to the female's antennae (van den Assem, Jachmann, et al., 1980; Ruther et al., 2010). Repeated head-nodding bouts are complemented by specific movements of the male's forelegs and antennae over the female's head (van den Assem, Jachmann, et al., 1980; van den Assem & Vernel, 1979; van den Assem & Werren, 1994). When the female shows receptivity by lowering her antennae and opening the genital orifice, the courting male moves backwards, and copulation follows. As males are usually smaller than females (Darling & Werren, 1990), there is a short time lag between the female opening the genital orifice and the male taking up the copulation posture on the female's abdomen. Frequently, a second or third male mounts the female together with the courting male and copulates with the female when she becomes receptive (van den Assem, 1996; van den Assem, Gijswijt, et al., 1980; van den Assem & Vernel, 1979). We hypothesise that this sneaking behaviour is exhibited only by subordinate males and that territorial males would gain copulations by honest courtship instead, that is, they would court the females themselves before copulation.

Territoriality and dominance–subordinance relationships are usually relatively stable against disturbances and over time (Fitzpatrick & Wellington, 1983; Kaufmann, 1983). We

investigated the stability of *Nv* territoriality across disturbances caused by newly emerging wasps as well as the stability of the system over time. We hypothesise that territorial males would (1) persist in the territorial position during the emergence of new males, (2) return to their territories after copulation with newly emerged females and (3) persist in their position for longer periods.

We also investigated *Nv* males with respect to factors determining and helping to maintain a male's territorial status. Van den Assem (1996) proposed that the male emerging first usually becomes the holder of the territory on the host. Two other studies pointed out the importance of body size for the outcome of male–male contests (van den Assem, Jachmann, et al., 1980; Tsai et al., 2014a; b). However, body size does not seem to result in fitness advantages for larger males in contest situations (Blaul & Ruther, 2012; Burton-Chellew, Sykes, et al., 2007; Moynihan & Shuker, 2011). We therefore performed additional experiments to test whether body size and emerging first are correlated with territoriality. We hypothesise that territorial males would be larger than subordinate males. In accordance with van den Assem (1996) we further hypothesise that males that emerged first from the host would become territorial.

Another factor known to determine the outcome of dominance–subordinance interactions and territorial disputes in various animal species is experience (Baker, 1983; Kaufmann, 1983). We hypothesise that the experience of being in the territorial position would affect the outcome of future territorial contests, that is, formerly territorial males should win contests against formerly subordinate males. Likewise, a lack of experience of territoriality should result in the loss of this advantage.

In addition, we investigated microcosm video recordings with respect to species-specific differences between *Nv* and *Ng* in the temporal pattern of emergence, copulation and dispersal. Owing to the high within-host mating rates in *Ng* (Drapeau & Werren, 1999; Giesbers et al., 2013; Leonard & Boake, 2006), we hypothesise that *Ng* females, unlike *Nv* females, would not mate or would be more reluctant to mate after emergence from the host. In accordance with Leonard and Boake (2006) we hypothesise that individuals of *Ng* would disperse faster (females) and in larger numbers (males) than those of *Nv*. We further hypothesise that if males of *Nv* dispersed they would wander off to establish a territory on another host from which females were about to emerge (van den Assem, Gijswijt, et al., 1980; King et al., 1969; Shuker et al. 2005). Information on species-specific characteristics can give a valuable background on which new hypotheses concerning the avoidance of reproductive interference between these two species can be built.

GENERAL METHODS

Strains, Rearing and Establishment of Groups

Experiments were performed with the *N. vitripennis* strain Phero01 and the *N. giraulti* strain NGVA2. Wasps were reared on freeze-killed pupae of the green bottle fly *Lucilia caesar* at 25 °C and under a 16:8 h light:dark regime. In each generation, several females were transferred to new petri dishes containing several fresh hosts. Hosts used in experiments were therefore likely to be parasitised by more than one ovipositing female. The basic observational units in all experiments were groups of wasps emerging from or having already emerged freely from hosts positioned inside an observation arena. For group preparation, single parasitised hosts from which wasps were about to emerge were taken from the breeding line and glued to pieces of ordinary print-out paper using nontoxic glue. A circle (35 mm diameter) was drawn around the host, marking an area close to the host (defined as 'vicinity of the host'). We checked for parasitoids in the hosts by illuminating the puparia with a cold light lamp, making wasp larvae, pupae and eclosed wasps visible from outside the puparium. During development, pupae of *Nasonia* pass through clearly distinguishable colour stages changing from complete white, white with red eyes, white with black head/thorax to complete black. Only hosts containing black pupae or already eclosed wasps were used for host preparation. Each arena was covered with the lower part of a plastic petri dish (85 mm diameter, 13 mm height). Prepared hosts were checked the next morning for emergence of wasps and bioassays were conducted when either one male or whole groups of males had emerged.

Microcosm Video Recording

Dynamics of territoriality, dominance structure and general temporal patterns of emergence and dispersal were observed in *Nv* and *Ng* in a microcosm approach in which we conducted extensive video recording of wasps emerging freely from single hosts. Recording was done in either a two-compartment arena consisting of an emergence site containing a host from which wasps would emerge (referred to as 'emergence site') and an oviposition site containing fresh hosts for oviposition (referred to as 'oviposition site') or a three-compartment arena including an additional compartment containing a second parasitised host from which wasps had not yet started to emerge (referred to as 'alternative site').

The two-compartment arena was composed of two rings of acrylic glass (62 mm diameter, 30 mm height) connected via an acrylic glass tube (10 mm diameter, 43 mm length). The three-compartment arena was composed of three rings of acrylic glass (70 mm

diameter, 10 mm height) connected via round acrylic glass tunnels (half-tubes; 10 mm diameter, 5 mm height) in such a way that wasps were able to move from each compartment to each of the other two compartments. The three-compartment arena was less high to prevent wasps from moving out of focus during video recording. During the experiments, arenas were put on ordinary print-out paper, covered with a glass plate and illuminated indirectly by two neon lamps placed on the table at both sides of the arena. Indirect illumination was achieved by setting up two pieces of cardboard painted with white acrylic paint next to the neon lamps directed to maximise reflection of light into the arena. By using indirect illumination, hard shadows were avoided. Video recording was done by means of two Canon EOS M cameras equipped with the free software enhancement Magic Lantern which allows for additional adjustments of the camera settings. One camera equipped with a Canon EF-M 18-55 mm zoom lens took overview video footage of the whole arena. The overview was later used to keep track of single wasps leaving or returning to the emergence site and to investigate the temporal pattern of dispersal. Simultaneously, a second camera equipped with a Tamron SP 90 mm F/2.8 macro lens took a close-up of the emergence site. The close-up allowed for the identification of more subtle behaviours such as male marking behaviour or aggressive interactions.

At the emergence site, wasps were allowed to emerge freely from a single host and male territoriality was investigated in detail. At the oviposition site, females were supplied with fresh hosts giving them the opportunity to switch to host-seeking and oviposition behaviour after mating. In addition, giving females the opportunity to leave the emergence site prevented them from disturbing dynamics between males and newly emerging females there. Fresh hosts were prepared by thawing frozen *L. caesar* puparia at 30 °C for 1 h. Previous studies have shown that this kind of host is readily accepted by *Nasonia* females for oviposition. To increase attraction of mated females to the oviposition site, 200 ng of dimethyldisulphide (DMDS) were applied to two discs of filter paper (5 mm diameter each) and put into the middle of the oviposition site in between the four fresh hosts. DMDS is a component of the odour of rotting meat and has been shown to attract mated *Nv* females (Frederickx et al., 2013). At the alternative site, males had the opportunity to hold a territory on a second host as an alternative behavioural strategy, if the emergence site became too crowded and the chance of mating there decreased. The alternative host was taken from the normal breeding line and contained wasps that were expected to emerge approximately 2 days later. Males of *Nv* can distinguish parasitised from non-parasitised hosts and prefer hosts from which wasps are about to emerge (King et al., 1969). We checked for parasites in

the alternative host by illuminating the puparium with a cold light lamp. Only hosts containing black wasp pupae were used as alternative hosts. All hosts in the arena were glued to the paper to avoid wasps moving them. A circle (35 mm diameter) was drawn around the host at the emergence site and at the alternative site marking an area close to the host (vicinity of the host).

For trials with *Nv*, several hosts were prepared as described above, but were painted carefully with a minimal amount of white acrylic paint before being glued to the printing paper. Painting of the hosts increased the contrast between the host and the wasps moving on the host, and made subtle behaviours such as male marking activity visible in the video footage. Painting neither impaired emergence from the host nor changed the wasps' behaviour after emergence (M. M. Mair, personal observation). The following morning after all other components of the observation arena were set up, one host from which one or two males had already emerged was chosen randomly and put into the emergence site by placing the arena over the paper on which the host was glued, positioning the host (and the already emerged male(s)) in the middle of the compartment. After the arena lid was closed, recording was started and continued as long as possible but no longer than 2100 hours. Continuous recording was achieved by setting an automatic record restart and frequently exchanging memory cards. Hosts were prepared daily for 2 months and video recording was conducted when a single male had emerged from at least one host. Recordings of hosts from which no further wasps emerged during the day were discarded. In total, 12 recording sessions with *Nv* were successful and used for analysis.

In trials with *Ng*, wasps emerged at a very fast rate. When prepared hosts were checked in the morning, complete groups of wasps had usually already emerged from the hosts. Thus, one host puparium containing eclosed adult wasps was chosen in the evening before expected emergence, painted carefully with white acrylic paint and glued to the paper in the emergence site. The developmental stage of the wasps inside the puparium was checked by illumination with a cold light lamp. After all components of the arena were set up, cameras were programmed to start recording automatically at 4 a.m. the next morning. Lights were programmed to turn on at the same time by means of a common timer switch. Recording was continued for about 4–5 h, until the memory cards were full. Hosts were prepared daily for 2 months. Recordings in which no wasps emerged from the host were discarded. In total, five recording sessions with *Ng* were successful and used for analysis.

For both species, a timetable was set up for each recording session noting the time and file name of each emergence (either female or male) and copulation event by rapid visual

scanning of the recorded close-up video files. Using this information, we then analysed subsamples from the video footage.

Statistical Analysis

All analyses were conducted in R v.3.3.2 (R Development Core Team, 2015). Generalised linear mixed models were fitted using the package 'glmmTMB' (Brooks et al. 2017). Because of the zero-inflated data structure, a negative binomial distribution was used to model count data. *P* values for fixed factors were gained via likelihood ratio tests using the 'drop1' function which compares, based on the Akaike information criterion (AIC), the original model with a reduced model dropping the respective fixed factor.

Ethical Note

Observational experiments with live insects are excluded from legislation in Germany. Insects were kept under near natural conditions on freeze-killed hosts. Live insects were observed in seminatural microcosm experiments. The number of replicates did not exceed the range necessary to enable solid statistical analysis.

TERRITORIALITY

Methods

To investigate territoriality and dominance in groups of males of *Nv* or *Ng* we took 2 min subsamples from the close-up video footage of the emergence site. Subsamples originating from the same recording session were separated by at least one emergence event (male or female). Subsamples were centred in between emergence events. Those containing only a single male started 5 min prior to the next emergence event. To limit the workload to a manageable amount, subsamples with groups consisting of more than eight males were discarded. According to van den Assem (1986), a transition from territoriality to scramble competition occurs at a group size of six males which is covered by the sampling. In total, 38 subsamples were taken from *Nv* and eight from *Ng* video footage.

Subsamples were cut from the video footage with the free software Avidemux version 2.6.8, stored as separate files and subsequently analysed using the software The Observer XT 11.5 (Noldus Information Technology Inc., Wageningen, The Netherlands). For each subsample, the time each male of the group spent on the host, in the vicinity of the host (within the inner circle drawn on the paper) and on the periphery (rest of the arena) was

recorded (see representative video sequence Movie S1 in the Supplementary material of the published article).

Each male was then classified as being either territorial (dominating the position on the host) or subordinate (nonterritorial, not dominating the position on the host). A male was classified as territorial if (1) it spent at least twice as long on the host as every other male in the group; (2) it spent at least 1 min on the host when no other male mounted the host; (3) it spent at least twice as long in the vicinity of the host (inner circle) as every other male when no male mounted the host. In groups in which two males shared the position on the host, that is, both spent twice as long as all other males on the host, both males were classified as territorial. All other males were classified as subordinate. The time males spent in each of the three locations (on the host, in the vicinity of the host, on the periphery) was visualised by means of separate bar plots for each subsample. The classification was used as a basis for the further investigation of behavioural differences between territorial and subordinate males.

Results

Males of *Nv* exhibited clear territoriality in terms of one or two males of the group dominating the position on the host (Fig. 1). In 35 of 38 samples, one male of the group was identified as territorial. In the remaining three samples, the territorial position was shared between two males. In *Ng*, a territorial male was determined in only one of eight samples.

DOMINANCE–SUBORDINANCE INTERACTIONS

Methods

All subsamples with groups consisting of two or more males were further investigated by noting for each male the number of won or lost aggressive interactions. An interaction was considered aggressive when it included behaviours such as wings turned into a vertical position, rapidly running or jumping towards another male and/or grabbing the other male while standing on top of it (for a detailed description of aggressive male–male interactions

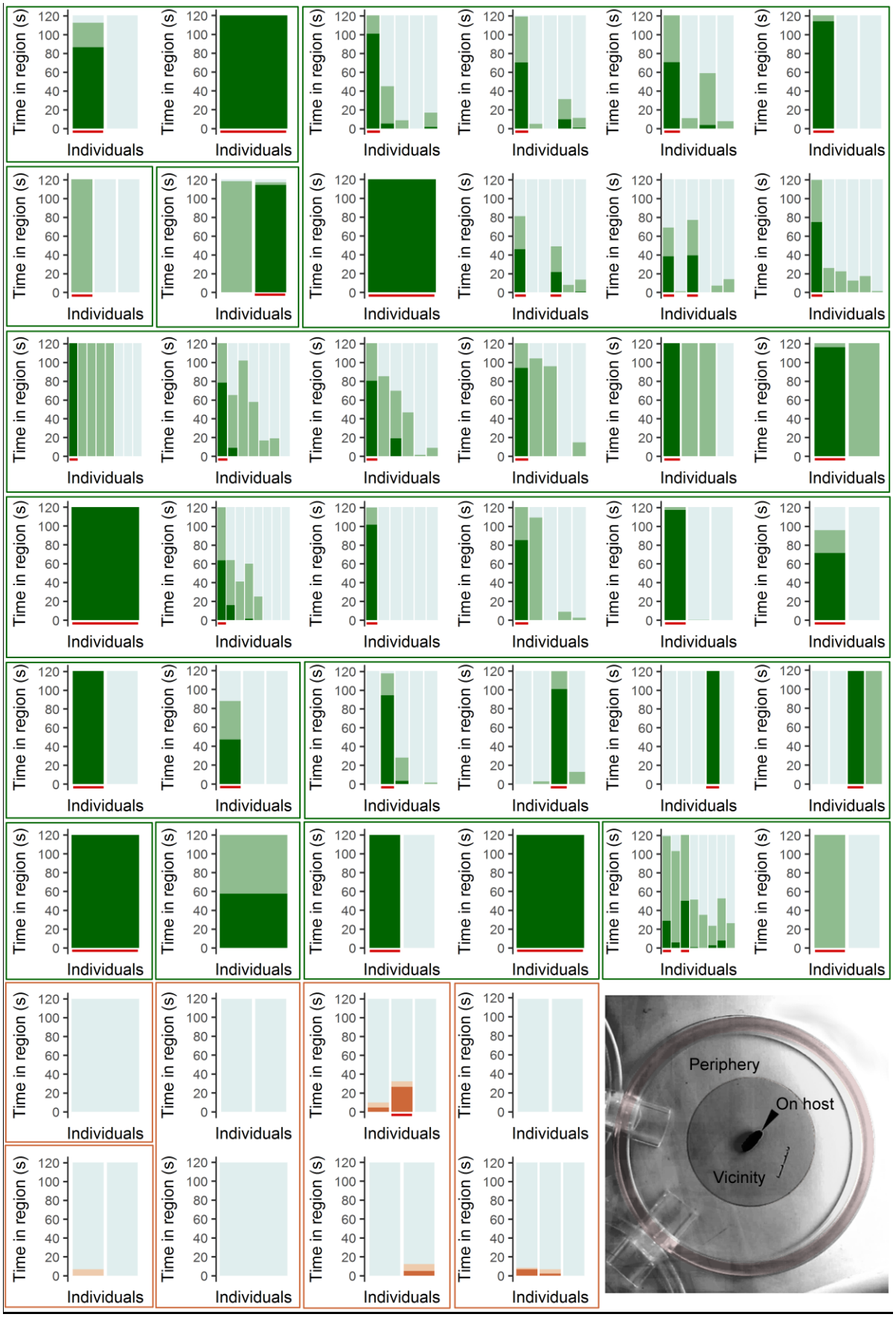

Figure 1 Results from observations of *N. vitripennis* (*Nv*, green) and *N. giraulti* (*Ng*, orange) male groups at the emergence site during 2 min subsamples taken from microcosm video recording sessions. Each panel

represents one group of males and each bar represents one male within each group. The figure shows the time that each male spent at three different regions: on the host (dark colour), in the vicinity of the host (medium colour) and on the periphery (light grey). Individuals are arranged randomly within each group. Samples belonging to the same recording session are surrounded by boxes (*Nv*, green; *Ng*, orange). The predefined ranges of the three regions are shown in the lower right corner. Individuals determined as being territorial are marked by red lines below bars.

see Leonard & Boake, 2006). An interaction was considered to be won by a male when he ran after the opponent at the end of the interaction or grabbed the opponent which crouched and remained still. An interaction was considered lost by a male when he ran away from the opponent or crouched against the ground at the end of the interaction. An interaction was considered to have ended when the two males separated, that is, they no longer showed any of the behaviours described above or when one of the males started an interaction with a third male.

The previously determined territorial status of each male was used to test whether territorial males win aggressive interactions more often than subordinate males. The number of aggressive interactions won by each male was analysed by fitting a generalised linear mixed model (glmm) with negative binomial distribution and log link function. Territorial status was included as a fixed factor and subsample, recording session and the total number of interactions were included as random effects. In addition, the difference between *Ng* and *Nv* in the total number of interactions per subsample was analysed by fitting a glmm with negative binomial distribution and log link function including species as a fixed factor and recording session and group size as random effects.

Results

In *Nv*, territorial males won interactions more often than subordinate males (negative binomial model (number of interactions won by territorial versus subordinate males): χ^2_1 =13.55, N = 16, P < 0.001; Fig. 2a). The total number of aggressive interactions per subsample did not differ between *Nv* and *Ng* (negative binomial model (total number of aggressive interactions in samples of *Nv* versus samples of *Ng*): χ^2_1 = 0.92, N_{Nv} = 30, N_{Ng} = 6, P = 0.34; Fig. 2b).

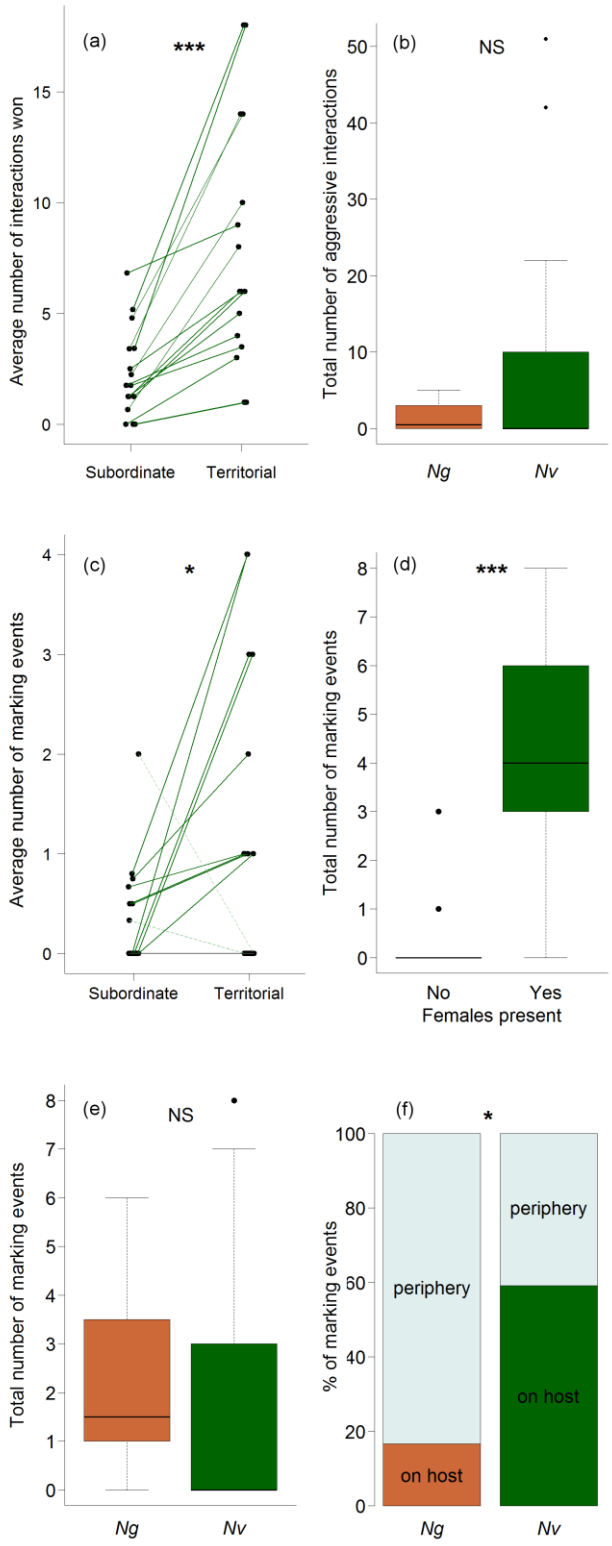

Figure 2 Results of dominance–subordinance interactions and male sex pheromone marking from the behavioural analysis of 2 min subsamples taken from microcosm video recordings of *N. vitripennis* (*Nv*, green) and *N. giraulti* (*Ng*, orange) male groups. (a) The average number of interactions won by territorial and subordinate males in groups of *Nv*; (b) comparison of the total number of aggressive interactions counted in groups of *Nv* and *Ng*; (c) the average number of marking events exhibited by territorial versus subordinate males in groups of *Nv*; (d) the number of marking events exhibited in *Nv* groups in the presence versus absence of females; (e) comparison of the number of marking events counted in groups of *Nv* and *Ng*; (f) comparison

between *Nv* and *Ng* in terms of the location where markings were applied. Lines in scatter plots (a, c) connect data points from the same subsample. Box plots display median (horizontal line within the box), lower and upper quartile (box margins), maximum/minimum range (whiskers; distance to median less than 1.5 x box height) and outliers (black dots; distance to median at least 1.5 x box height). (a–e) Negative binomial model and (f) binomial model: $*P < 0.05$; $***P < 0.001$.

MARKING BEHAVIOUR

Methods

The correlation between territoriality and marking behaviour was investigated by noting for each subsample the number of marking events for each male. The previously determined territorial status of each male was used to test whether territorial males show marking behaviour more often than subordinate males. The number of marking events of each male was analysed by fitting a glmm with negative binomial distribution and log link function including territorial status as a fixed factor and subsample and recording session as random effects. Because males are known to increase marking after having had contact with females (van den Assem, 1996; van den Assem, Jachmann, et al., 1980; Barrass, 1969; Steiner & Ruther, 2009b), marking activity was compared between subsamples with females present at the emergence site and those where females were absent. The number of marking events in each subsample was analysed by fitting a glmm with negative binomial distribution and log link function including female presence (yes/no) as a fixed factor and recording session and group size (i.e. number of males present) as random effects.

In addition, the difference between *Ng* and *Nv* in the number of marking events and in the location where marking was exhibited (on the host, in the vicinity of the host, on the periphery) was investigated. The number of marking events in each subsample was analysed by fitting a glmm with negative binomial distribution and log link function including species as a fixed factor and recording session and group size as random effects. As no marking behaviour was performed in the vicinity of the host, a glmm with binomial distribution and logit link function was fitted to the number of marking events exhibited in each subsample on the host and on the periphery including species as a fixed factor and recording session and group size as random effects.

Results

In *Nv,* territorial males marked the substrate more often than subordinate males (negative binomial model (number of marking events exhibited by territorial versus subordinate males): $\chi^2_1 = 6.35$, $N = 30$, $P = 0.012$; Fig. 2c), and marking behaviour was

exhibited more often when females were present (negative binomial model (total number of marking events in samples with females versus samples without females): $\chi^2_1 = 21.03$, $N_{\text{females}} = 9$, $N_{\text{no females}} = 21$, $P < 0.0001$; Fig. 2d).

Males of *Ng* marked the substrate as often as males of *Nv* (negative binomial model (number of marking events in samples of *Nv* versus samples of *Ng*): $\chi^2_1 = 1.18$, $N_{Nv} = 36$, $N_{Ng} = 8$, $P = 0.28$; Fig. 2e). The two species differed in the location where pheromone marking was exhibited. While *Ng* concentrated marking activities on the periphery of the arena, more than half of all marking events in *Nv* happened on the host (binomial model (number of marking events on the host/on the periphery in samples of *Nv* versus samples of *Ng*): $\chi^2_1 = 6.42$, $N_{Nv} = 36$, $N_{Ng} = 8$, $P = 0.011$; Fig. 2f). No marking was performed in the vicinity of the host.

THE VALUE OF THE RESOURCE

Removal Experiment

Methods

Wasps were allowed to emerge freely from hosts prepared as described above. In the morning, hosts were checked and all emerged females were removed. After removal of the females, male groups were allowed to settle again for at least 30 min. To identify the territorial male of a group, the group was observed carefully for 1 min. The male spending most of the time on the host (at least 30 s) and winning aggressive interactions with other males was termed territorial. If no territorial male could be determined, the group was left for at least 5 min before checking again. If determination of a territorial male was still not possible, the group was discarded. In groups containing a territorial male, he was removed carefully from the arena and the remaining males were allowed to establish a new dominance structure. After 4 min the group was observed again for 1 min and, if possible, a new territorial male was determined. For each replicate ($N = 39$) the group size (number of males in the group prior to removal of the territorial male) and whether a new territorial male could be determined were recorded. According to van den Assem (1986), territoriality in *Nv* male groups breaks down in groups of six or more males. The frequency of successful replacement of the territorial male after removal was thus compared between smaller (up to five males) and larger (more than five males) groups with a Fisher's exact test.

Results

In 31 of 39 samples (79.5%), the position on the host was successfully taken over by another *Nv* male within 5 min after removal of the territorial male from the group. The territorial male was replaced less often in larger groups (6–15 males; 57.1%) than in smaller groups (2–5 males; 92.0%; Fisher's exact test: $N_{large} = 14$, $N_{small} = 25$, $P = 0.016$).

Mating Success

Methods

Close-up video footage of *Nv* at the emergence site was analysed by observing the territorial male in time periods surrounding female emergence events. Each observation started 1 min before the emergence of a female wasp. Within this minute, the territorial male in the group was determined following the criteria explained above. Observations lasted until all males had dismounted the female plus 2 min to check for multiple mating. If one or more males mounted the female at this point, the observation was continued until all of the males had dismounted again. During the observation, the territorial male was followed carefully. We noted whether the territorial male or one of the subordinate males succeeded in copulating with the female (first mating). In addition, we noted whether the copulation was achieved by sneaking, that is, the male copulating was not the male that courted the female, or by honest courtship, that is, the male that courted the female also succeeded in copulating. In total, 25 female emergences in seven recording sessions were observed. These included samples in which a new female emerged while the territorial male was still engaged in courtship with a female that had emerged earlier, a situation that could impair the mating success of territorial males relative to subordinate males under natural conditions.

For each recording session, the number of females mating with the territorial or one of the subordinate males was counted. To test whether territorial males were more successful in mating than subordinate males, a glmm with binomial distribution and logit link function was fitted to the number of females mated/not mated, including territorial status as a fixed factor and recording session as a random effect. In this analysis, the mating success of territorial males was compared to the mating success of all subordinate males taken together. The mating success of territorial males was therefore also compared to the expected mating success assuming a random mating structure. For this purpose, the number of females expected to mate with each male in the group under conditions of random mating was calculated for each recording session by dividing the number of emerged females by the

number of males present in the group. To test whether territorial males succeeded in mating more often than expected by chance, a glmm with binomial distribution and logit link function was fitted to the number of females mated/not mated, including observed/expected as a fixed factor and recording session as a random effect. The difference in the sneaking behaviour between territorial and subordinate males was analysed by fitting a glmm with binomial distribution and logit link function to the number of copulations gained by sneaking/honest courtship including territorial status as a fixed factor and recording session as a random effect.

Results

Territorial *Nv* males had no mating advantage over subordinate males. Territorial males were the first males to copulate with newly emerging females as often as subordinate males (binomial model (number of females in each recording session that mated with the territorial male versus one of the subordinate males): $\chi^2_1 = 0.78$, $N = 7$ (recording sessions), $P = 0.38$; Fig. 3a). In addition, the mating success of territorial males did not differ from that expected by chance considering the total number of males present in the group (binomial model (number of females in each recording session that mated with the territorial male versus expected number of females that would have mated with the territorial male under random mating): $\chi^2_1 = 0.48$, $N = 7$ (recording sessions), $P = 0.49$; Fig. 3b). Males differed in the behavioural strategy used to achieve copulation success. While all territorial males courted the females themselves before mating, half of the subordinate males succeeded in mating by sneaking in when females opened their genital orifice after having been courted by another male (binomial model (number of copulations gained by honest courtship or sneaking, respectively, in territorial versus subordinate males): $\chi^2_1 = 10.0$, $N = 7$ (recording sessions), $P = 0.002$; Fig. 3c).

STABILITY OF THE DOMINANCE STRUCTURE

Methods

The stability of the dominance structure in *Nv* against disturbances was investigated in the close-up video footage by observing male groups before and after emergences of new female and male wasps. Female emergence events were investigated again in detail following the territorial male and noting for each sample whether the territorial

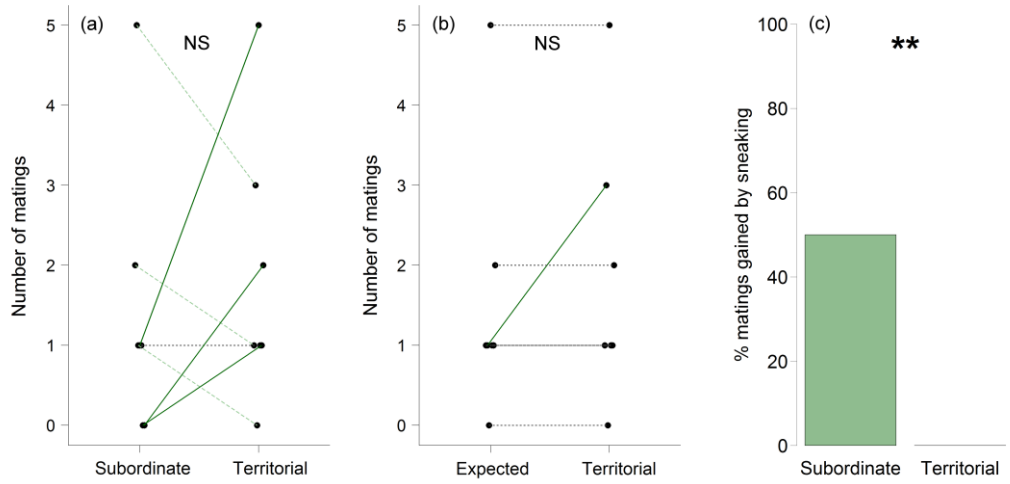

Figure 3 (a) The number of first matings gained by subordinate and territorial males in groups of Nv observed in microcosm experiments. (b) Comparison of the mating success of territorial males with that expected by chance considering the size of the group. (c) Comparison of the mating strategy (matings gained by either sneaking or honest courtship) of territorial and subordinate males. Binomial model: **P < 0.01.

male (1) returned to the host, (2) returned either before or after the previously emerged female mated (irrespective of the status of the male that mated) and (3) succeeded in taking back the territorial position on the host. A male was considered having taken back the position on the host when he successfully chased away opponents present upon return and when he was able to defend his position for at least 1 min. Samples in which the territorial male was still distracted by courting a female that had emerged earlier were discarded. Two samples in which the female moved out of focus were also discarded. In total, 19 female emergences were investigated.

For male emergences, subsamples were taken from the close-up video footage starting 1 min before each male emergence and lasting 3 min in total. If a second new male emerged from the host during this 3 min period, the sample was discarded, as the second emergence event could have further disturbed the group. During the first minute (prior to the emergence of the new male), the territorial male was determined. If no territorial male could be determined, the sample was discarded. After emergence of the new male, the group was given 1 min to settle again during which time the territorial male was followed carefully. During the third minute, the territorial male was observed as to whether he was still in the territorial position defending the host against opponents or not. In total, 22 male emergence events were investigated.

To investigate the stability of the dominance structure over time, groups of males were observed over longer time periods. For this purpose, 1 h subsamples were taken from the

close-up video footage of *Nv* and the number of events where one male challenged the territorial male and succeeded in taking over the territorial position on the host were counted. Subsamples were taken from time periods in which no other disturbances such as emergences or copulations occurred for at least 1 h. Subsamples started 5 min after the last emergence event or, in the case of female emergence, after the respective female consented to mating. During the first minute of the observation, the initial territorial male was determined and subsequently followed carefully. A challenging male was considered having taken over the territorial position successfully when he exhibited dominance behaviour towards the former territorial male, took the position on the host and was able to defend this position for at least 1 min. After a take-over event, the new territorial male was followed. In addition, the number of males present in each group was noted. In total, 10 subsamples from nine recording sessions were investigated.

If the territorial structure in *Nv* is stable, it should resist disturbances by later emerging wasps as well as persist over longer time periods. Owing to the low sample sizes and low variation in the response variables, no statistical tests were performed on the data.

Results

Emergence events did not disturb the territorial structure in groups of males. All observed territorial males returned to the host after having mounted and eventually courted newly emerged females ($N = 19$). In addition, all these males succeeded in taking back the territorial position on the host upon return and were able to defend their position against competitors for at least 1 min. Four of the males returned to the host before the emerged female consented to mating, and 15 territorial males returned after having copulated successfully. When groups were disturbed by newly emerged males, the territorial male persisted in his position in 21 of 22 samples (95%).

In the 1 h observations, the territorial male persisted in his position in eight of 10 samples (80%). Group sizes in samples without take-over events ranged from two to five males. The two samples in which take-over events were observed (three such events each) consisted of eight and 10 males, respectively.

FACTORS CORRELATED WITH MALE TERRITORIALITY

Body Size

Methods

In an additional experiment, the correlation between body size and territoriality in *Nv* was investigated by measuring the size of males in groups. Hosts were prepared as described above and wasps were allowed to emerge freely. For each group, the territorial male was determined as described above. If no territorial male could be determined, the group was given at least 5 min before being checked again. If determination of the territorial male failed a second time, the group was discarded. The territorial male and each of the subordinate males were put into 1.5 ml microcentrifuge tubes separately, labelled with group number and territorial status, and freeze-killed at -20 °C. The head width of each male was subsequently measured using a Keyence VHX 600 digital microscope (Keyence Deutschland GmbH, Neu-Isenburg, Germany). For measurements, the males were laid on their backs and maximum head width was measured from a ventral view. The average of three measurements was calculated and taken as a proxy for male body size (Blaul & Ruther, 2012). In total, 35 wasp groups were measured.

To test whether territorial males are larger than subordinate males, the average head width of all subordinate males belonging to the same group was calculated and compared to the size of the respective territorial males with a Wilcoxon signed-ranks test. In addition, the number of samples in which the territorial male was the largest male of the group was compared to the summed probabilities of being the largest male of the group by chance with a binomial test. For example, in a group of five, each male has a probability of 0.2 of being the largest male of the group by chance. If territoriality is not correlated with male body size, the territorial male is thus expected to be the largest male in two of 10 such groups (0.2 times 10). Summed probabilities were rounded to the nearest integer before analysis.

Results

Territorial males were larger than average subordinate males (Wilcoxon signed-ranks test: $T = 473$, $N = 35$, $P = 0.009$). In addition, the territorial male was the largest male of the group in 17 samples, which is more often than expected by chance considering the number of males present in each group (territorial male expected to be the largest male by chance in nine samples; binomial test: $N = 35$, $P = 0.003$).

Emerging First

Methods

The hypothesis that males emerging first become territorial was investigated. Hosts of *Nv* were prepared as described above. In the morning, hosts from which only a single male had emerged were chosen for experiments. The male was removed from the host and carefully marked by applying a spot of nontoxic red paint on the thorax or abdomen using a fine needle. The male was subsequently put back into the arena and allowed to settle. After 1, 5 and 24 ± 3 h, marked males were observed for 1 min and their territorial status was recorded. In addition, the number of emerged males and females was noted. In total, nine males were marked and observed. Owing to the low sample size, results are reported descriptively.

Results

In all nine replicates, males emerging from the host first became territorial. One hour after being marked, eight of nine males were positioned on the host. No other wasps had emerged at that time. After 5 h, seven marked males were dominating the position on the host, including the only replicate in which one additional male and one female had already emerged. After 24 h, all eight remaining marked males were determined as being territorial, with groups consisting of one to five males (median 2) and zero to five females (median 2). In one sample, the marked male was not found again in the arena after 24 h.

Body Size and Order of Emergence

Methods

In an additional experiment, the correlation between body size and early emergence from the host was investigated in *Nv*. Hosts were prepared as described above. In the morning, several hosts were observed simultaneously and single males emerging from the hosts were put into 1.5 ml microcentrifuge tubes separately, labelled with host number and position of the respective male in the order of emergence and killed by freezing. Emerging females were removed. When wasps had already emerged in the morning, all these wasps were removed. The number of male wasps removed was counted to allow us to determine correctly the position in the order of emergence of later emerging males. The head width of all collected males was measured as described above. The correlation between early emergence and head width was investigated by Spearman rank correlation.

Results

Males emerging from the host earlier were larger than males emerging later (Fig. 4).

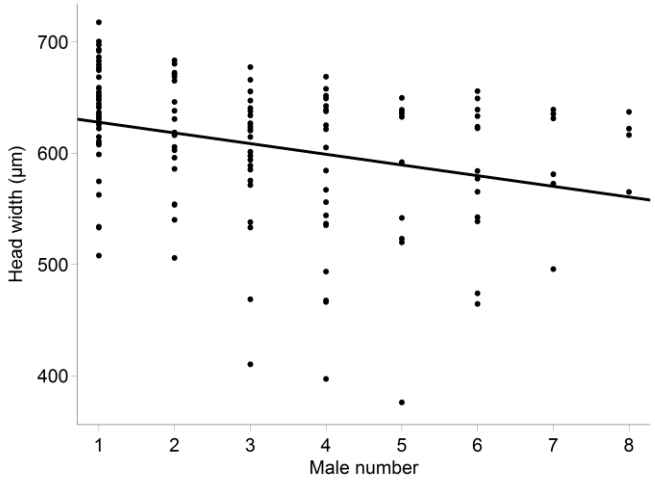

Figure 4 Correlation between the order of emergence and the head width of *Nv* males that emerged naturally from their hosts (Spearman rank correlation: $r_S = -0.35$, $N = 138$, $P < 0.0001$).

Effect of Experience: Isolation Experiment

Methods

The role of experience was investigated in another experiment by isolating either a territorial or a subordinate male from a group and testing whether the isolated male was able to become territorial (again) after returning to the same group. If having experienced being territorial does have an effect, territorial males should become territorial again more often than subordinate males that had been isolated for the same amount of time. In addition, prolonged isolation of the territorial male from the group to prevent him experiencing being territorial should lead to a decrease in the ability to take back the territory after return.

Hosts of *Nv* were prepared as described above. In the morning, hosts were checked, all females were removed from the groups and males were allowed to settle again for at least 30 min. Male groups were then used in bioassays. The territorial male of a group was determined as described above. If no territorial male could be determined, the group was given at least 5 min before being checked again. If determination failed a second time, the group was discarded. After determination of the territorial male, one male of the group was carefully removed from the group and put into a 1.5 ml microcentrifuge tube for isolation. After isolation, the isolated male was reintroduced to the group by placing it on the paper on

the periphery of the arena from which he originated. The isolated male was followed for 4 min and his territorial status was subsequently determined by observing him for 1 min. The territorial status of the male was noted. In total, 20 replicates were conducted for each of three treatments. In treatment 1 the territorial male was isolated for 60 s. In treatment 2 the territorial male was isolated for 900 s. In treatment 3, the territorial male was removed from the group, and a random subordinate male was taken from the periphery of the arena and isolated for 1 min. The assignment of groups to treatments and the order of treatments were randomised.

The number of males returning to territorial status (or taking over the territorial status in case of isolated subordinate males) after isolation was compared between treatments by means of Fisher's exact tests. *P* values were corrected according to Benjamini–Hochberg (1995) to account for multiple comparisons.

Results

Territorial males that had been isolated from the group for 60 s took back the territorial position in 15 of 20 samples. Subordinate males isolated for the same amount of time achieved the territorial position less often than territorial males (five of 19 samples; Fisher's exact test: $P = 0.012$; Fig. 5a). One focal subordinate male moved into the host during observation. This sample was ignored in the analysis. Territorial males isolated for 900 s took back the territorial position as often as those isolated for 60 s (eight of 20 samples; Fisher's exact test: $P = 0.080$). The success rate of territorial males isolated for 900 s was not significantly different from that of subordinate males (Fisher's exact: $P = 0.50$).

Effect of Experience: Contests

Methods

The role that experience plays in *Nv* territoriality was investigated further by testing territorial and subordinate males originating from different male groups together in contest situations. Hosts were prepared as described above on 2 consecutive days (day 1 and 2).

Hosts prepared on day 1 were used to set up observational arenas 2 days later (day 3). On the morning of day 3, all wasps that had emerged freely from these hosts were removed. Each arena therefore consisted of an empty host glued to paper and covered by the bottom of a plastic petri dish. Hosts prepared on day 2 were checked on day 3, all females were removed, and males were given at least 30 min to settle before being used in experiments.

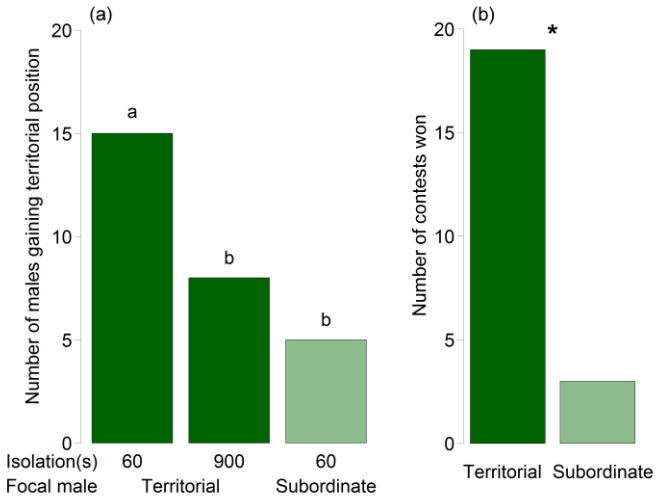

Figure 5 (a) Results of the isolation experiment showing the number of males that were able to take back (focal male territorial) or take over (focal male subordinate) the territorial position on the host after returning to the male group from which they had been isolated for 60 or 900 s. Different letters indicate significant differences: Fisher's exact tests: $P < 0.05$. (b) Outcome of territoriality contests on unknown empty hosts between two males at a time originating from two different male groups in which they had experienced being either territorial or subordinate. Binomial test of difference between observed and expected values: ***$P < 0.001$.

Two hosts were then chosen randomly and the territorial male was determined in both groups simultaneously. From one group the territorial male was removed and put into a 1.5 ml microcentrifuge tube. From the second group a haphazardly chosen subordinate male was taken from the periphery of the arena and also put into a microcentrifuge tube. Both males were then released onto the periphery of the observational arena simultaneously by putting the opened tubes upside down onto the paper, letting the males move to the paper, removing both tubes at the same time and closing the arena. One male was placed on the right side and the other on the left side of the arena to avoid immediate contact between the males after release. Assignment of males to the left or right side was randomised. The former territorial male was followed carefully for 4 min and his territorial status was determined for 1 min. The two males were finally put into two separate microcentrifuge tubes, freeze-killed and their head-widths were measured as described above. Each arena was used only once. In total, 30 replicates were performed.

To test whether experience affects the outcome of territoriality contests, the number of contests won by former territorial and subordinate males was compared to an expected 50% chance of winning via a binomial test. As the male groups had emerged naturally from hosts, we were not able to control for body size in these contests. Body size measurements were therefore used afterwards to test whether former territoriality (i.e. having experienced being

territorial) or the outcome of the contests (i.e. winning contests) was correlated with body size using Wilcoxon signed-ranks tests.

Results

Previous experience of territorial status was important for *Nv* males to achieve territorial status in contests on unoccupied hosts. In 19 of 22 samples, the former territorial male gained the territorial position on the host and defended this position against the former subordinate male. This was more often than what would have been expected by chance (binomial test: $P < 0.001$; Fig. 5b). In the contests, former territorial males were not larger than former subordinate males (Wilcoxon signed-ranks test: $T = 77$, $N = 22$, $P = 0.11$). In addition, contest winners were not larger than losers (Wilcoxon signed-ranks test: $T = 150$, $N = 22$, $P = 0.46$). In eight samples, none of the two males established clear territoriality within 5 min.

EMERGENCE, COPULATION AND DISPERSAL PATTERNS

Methods

For both species the temporal pattern of emergence, copulation and dispersal was investigated further by additional quantitative analysis of the video recordings. Characteristics were compared between the two species. The order of emergence of males and females was noted for each recording session and, owing to the low sample size, compared between the two species descriptively. Sessions with hosts from which no females emerged were excluded from analysis. The temporal pattern of emergences was investigated by calculating the time between emergence events. Sessions with hosts from which only males emerged were included here. Time between emergences was averaged over recording sessions and compared between the two species by a Mann–Whitney *U* test.

The pattern of copulation and female dispersal was investigated by following individual females carefully after emergence. Observation of a focal female was stopped when she left the emergence site. For each female we noted whether she showed receptivity and thus consented to copulation or not. Multiple mating of females was also noted. To test whether females of *Nv* consent to copulation at the emergence site more often than females of *Ng*, a glmm with binomial distribution and logit link function was fitted to the binary response mated/not mated including species as a fixed factor and recording session as a random effect. To check whether the low copulation frequency in *Ng* was connected to the reluctance of

males to start courtship we additionally noted for each female whether she was courted by at least one male before leaving the emergence site or not. In addition, for each female the time between emergence and (first) copulation and the time between emergence and leaving the emergence site was measured. Times were averaged over recording sessions and compared between the two species by Mann–Whitney *U* tests.

The temporal pattern of dispersal of males and females was investigated further by scan sampling of the overview video footage. The number of females and males present in each of the three compartments of the arena (emergence site, alternative site and oviposition site) was counted at 0, 4, 8, 16, 32, 64, 128 and 256 min after the first female had emerged. A geometric sampling sequence was used here to account for both the rapid emergence and dispersal pattern in *Ng* and the slower pattern in *Nv*. The proportion of females and males, respectively, present in each compartment at each time point was calculated and compared between the two species by Mann–Whitney *U* tests. The low sample sizes, particularly at later time points, did not allow for more elaborate modelling and restricted statistical tests to time points 4, 8, 16 and 32 min. In addition to wasp counts, the position and behaviour of females at the oviposition site (either ovipositing on the fresh hosts or not) and the position of males at the alternative site (to check for potential territoriality on the alternative host) were noted.

Results

The two species differed in the order of emergence of females and males from the host. While in *Nv* all wasps emerging first were males ($N = 7$), in *Ng* all wasps emerging first were females ($N = 4$; Fig. 6a, b). Additionally, in *Ng*, there were always more females than males present in the arena over the course of emergences, while in *Nv* more males than females were present over the first 10 emergences. The time between emergences (irrespective of the sex of the emerging wasps) was longer in *Nv* than in *Ng* (data averaged over each recording session; median (minimum, maximum)$_{Nv}$ = 601 (57.7, 6590) s, median (minimum, maximum)$_{Ng}$ = 62.4 (37.6, 141.9) s; Mann–Whitney *U* test: $U = 3$, $N_{Nv} = 8$, $N_{Ng} = 5$, $P = 0.011$; Fig. 6c).

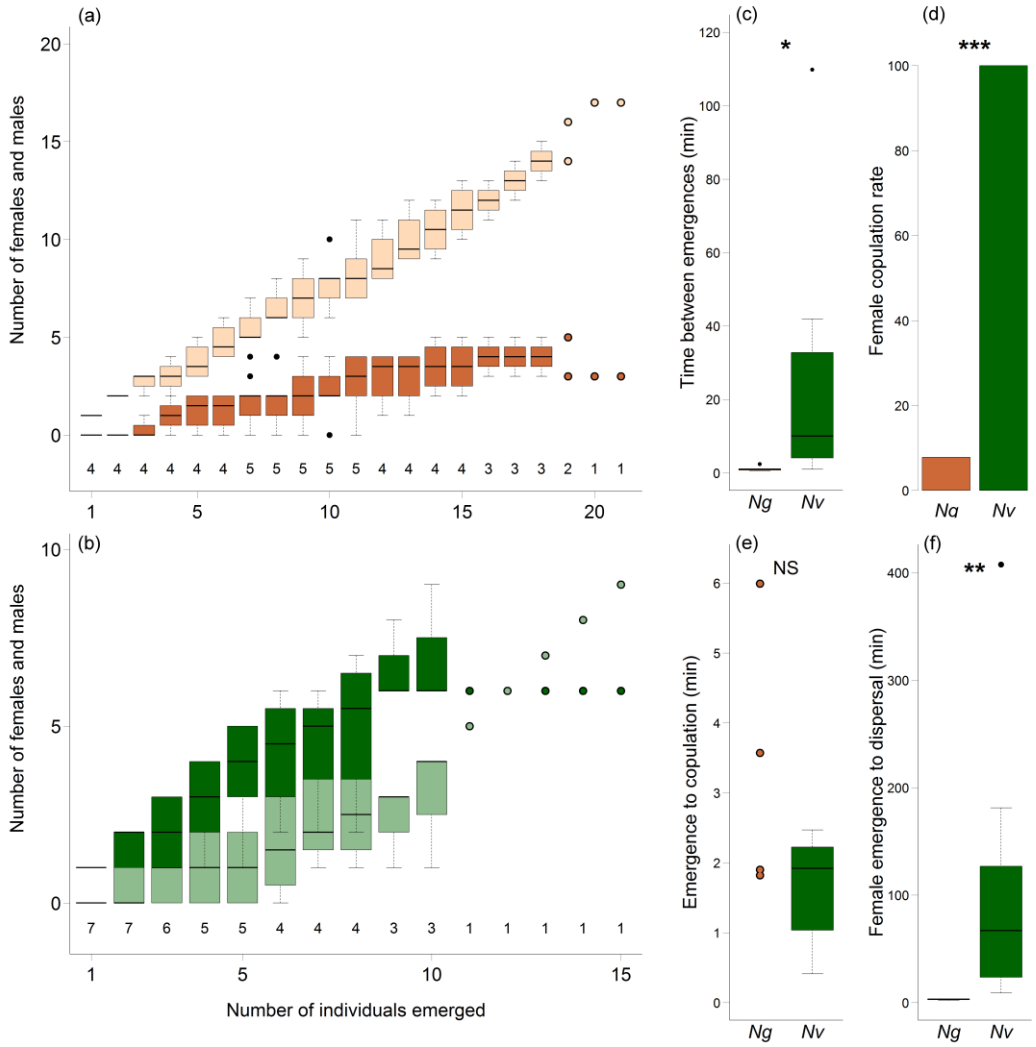

Figure 6 (a, b) Species-specific differences in the pattern of emergence of females and males, (c) the temporal pattern of emergence (females and males combined), (d) female copulation rate at the natal host patch after emergence from the host and the temporal pattern of (e) copulation and (f) dispersal of females. The total number of females (light boxes) and males (dark boxes) present in the arena over the course of the microcosm video recordings is shown for *N. girault*i (a, orange) and *N. vitripennis* (b, green) separately. Sample sizes are given below boxes. Owing to the low sample sizes at higher wasp numbers in the emergence pattern data (a, b) and the low sample size of the time between female emergence and copulation in *N. girault*i (e) data are displayed by single dots instead of a box plot. Box plots display median (horizontal line within the box), lower and upper quartile (box margins), maximum/ minimum range (whiskers; distance to median less than 1.5 x box height) and outliers (black dots; distance to median at least 1.5 x box height). Mann–Whitney *U* test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

In *Nv*, all 25 females emerging during recording sessions mated before leaving the emergence site. Seven females mated with two males, and one female mated with three males, two of which copulated with the female consecutively while the genital orifice remained open during the first mating event. In *Ng*, although 25 of 64 females were courted, only five of them consented to mating before leaving the emergence site (binomial model (mating frequency of *Ng* versus *Nv* females): $\chi^2_1 = 24.72$, $P < 0.0001$; Fig. 6d). Averaged

over recording sessions, females of *Ng* and *Nv* did not differ in the time between emergence and copulation (Mann–Whitney *U* test: $U = 20$, $N_{Nv} = 7$, $N_{Ng} = 4$, $P = 0.32$; Fig. 6e). Females of *Ng* dispersed to other compartments after emergence faster than females of *Nv* (Mann–Whitney *U* test (time lags averaged over each recording session): $U = 0$, $N_{Nv} = 7$, $N_{Ng} = 5$, $P = 0.003$; Fig. 6f).

Females of both species dispersed to both the alternative site (containing a parasitised host as an opportunity for males to establish a territory apart from the emergence site) and the oviposition site (containing fresh pupae for females to oviposit in; Fig. 7a–c). Females of *Ng* were found in equal numbers in the alternative site and the oviposition site, where they were mainly running or sitting on the arena lid, wall and floor, frequently changing compartments. No female of *Ng* was observed ovipositing during scan sampling. Four of the five recording sessions in *Ng*, however, lasted for less than 1 h and oviposition may have occurred after that. In contrast, although females of *Nv* were also found at the alternative site, at the end of the scan sampling period (256 min after the emergence of the first female) most females of *Nv* were found at the oviposition site where they usually sat on the fresh hosts and started ovipositing. While some males of *Ng* dispersed to both the alternative and the oviposition site, none of the males of *Nv* left the emergence site during scan sampling (Fig. 7d–f). No male was found sitting on or defending the host at the alternative site.

DISCUSSION

Nv and Ng exhibit different behavioural strategies at the natal host patch, including differences in territoriality and dominance structure, marking activity, order and temporal pattern of emergence and dispersal. Males of *Nv* clearly meet the concept of territoriality. One male, or less frequently two males, occupied the territorial position on the host, the valuable resource from which females were about to emerge. The territory was defended aggressively against competitors and marked with the abdominal sex pheromone. The holder of the territory was able to stay in his position over prolonged periods, persisted when new males emerged from the host and returned to his territory after having courted and eventually mated with a newly emerged female. When the territorial male was removed, his position was quickly taken over by another male of the group. Consistent with earlier anecdotal descriptions (van den Assem, 1996; van den Assem, Gijswijt, et al., 1980; van den Assem,

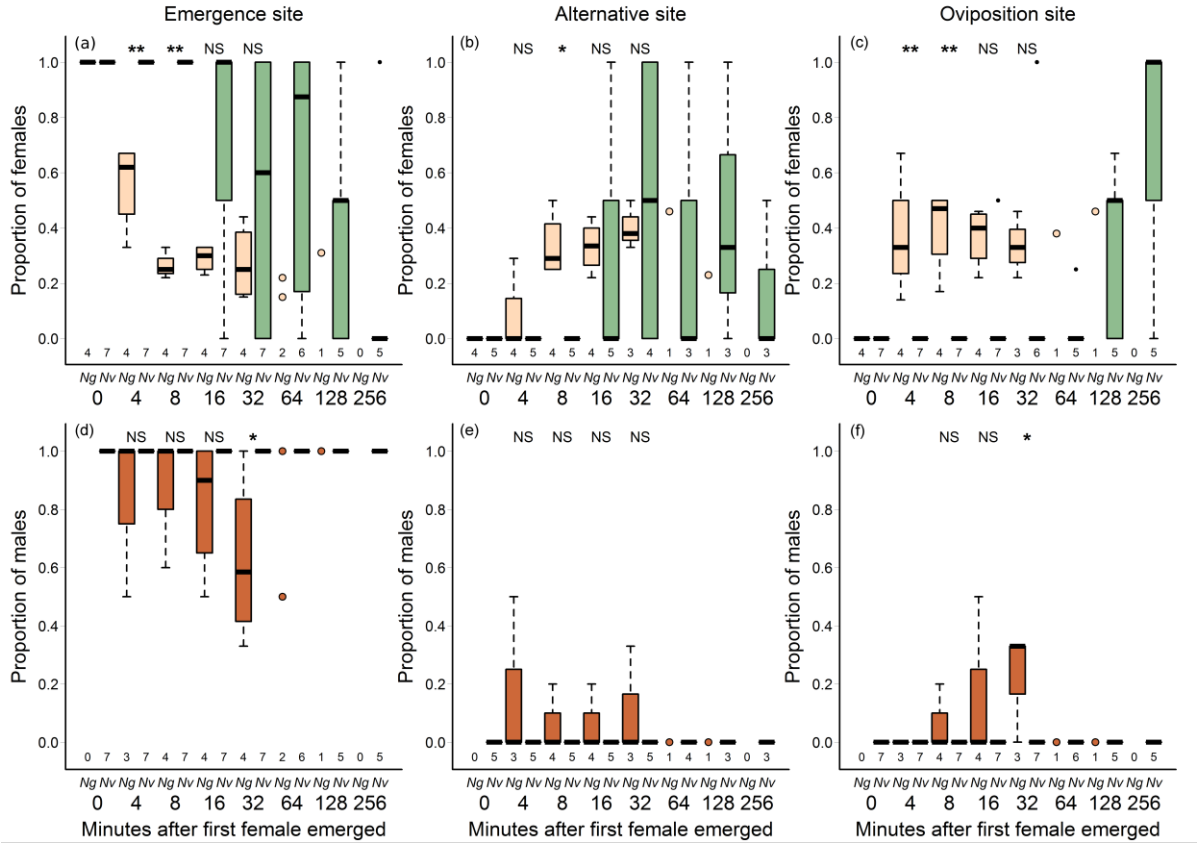

Figure 7 Temporal pattern of dispersal of (a–c) females and (d–f) males of *N. vitripennis* (*Nv,* green) and *N. girault*i (*Ng,* orange) from the natal host patch (emergence site; a, d) to the alternative behavioural strategy site (alternative site; b, e) and the oviposition site (c, f) during video recordings of freely emerging wasps in a microcosm set-up. The proportions of females (dark boxes) and males (light boxes) that were present in each of the three compartments are displayed. Sample sizes are given below boxes. Box plots display median (horizontal line within the box), lower and upper quartile (box margins), maximum/minimum range (whiskers; distance to median less than 1.5 x box height) and outliers (black dots; distance to median at least 1.5 x box height). Coloured dots are displayed instead of boxes for sample sizes lower than three. Mann–Whitney *U* test: $*P < 0.5$; $**P < 0.01$.

Jachmann, et al., 1980; King et al., 1969), these findings establish a more solid scientific basis for understanding *Nv* male territoriality.

Although territorial males are often larger than subordinate males, emerging first and experience are more important than body size for males to become territorial and stay in the territorial position. In our experiments, territorial males were larger than average subordinate males, and were the largest males of the group more often than expected by chance. In addition, body size has been found to play an important role in contest situations in naïve males that had been isolated prior to eclosion (Tsai et al., 2014b). However, body size and early emergence are correlated in *Nv*. Moynihan & Shuker (2011) showed that larger males develop faster, eclose earlier and, thus, emerge earlier from the host puparium. In contest

situations in the presence of females, males emerging first were able to father more offspring, irrespective of their body size. In addition, two other studies with naïve males also found no evidence for an effect of body size on mating success (Blaul & Ruther, 2012; Burton-Chellew, Sykes, et al., 2007). However, these previous experiments used males that had never experienced dominance–subordinance interactions and were conducted in the absence of a host puparium, a valuable resource worth defending. We therefore performed an experiment to investigate the effect of experience in a contest situation where two males that had experienced being either territorial or subordinate competed for a host. In this experimental set-up, too, body size was not correlated with the outcome of male–male contests. In contrast, males that had experienced being territorial were able to establish territoriality on the host in almost all samples. A second experiment conducted to investigate the effect of early emergence on the territorial ability of males showed that males emerging first became territorial and were able to persist in this position over a prolonged time. We therefore conclude that, although body size does play a role in naïve males, this effect is less important than that of early emergence and experience in freely emerging groups.

The importance of experience and arriving early at a territorial area for the establishment of territoriality has been shown in various animal taxa (Morbey & Ydenberg, 2001; Takeuchi & Honda, 2009). In many of these species, the resident usually wins disputes with competitive intruders (Fitzpatrick & Wellington, 1983; Kemp & Wiklund, 2004). This prior residency advantage and the effect of experience both involve memory of earlier incidents and thus learning (Benelli et al., 2015; Hsu et al., 2006; Stamps & Krishnan, 1999, 2001). Learning makes a territorial system highly flexible, as the response of an individual to a specific situation can be changed with every new experience made. The intertidal owl limpet, *Lottia gigantea*, for example memorises outcomes of earlier aggressive interactions and integrates these memories into behavioural reactions in future contests (Wright & Shanks, 1993). Individuals that have repeatedly experienced defeat react submissively in encounters with competitors whereas individuals that have won earlier contests react aggressively. Furthermore, these learned reactions are reversible by simply adding contrary experiences to the individuals' memories. In isolation experiments with *Nv*, we found evidence that the experience of being territorial strengthens the future ability of territorial males to persist in the territorial position whereas periods of isolation from the valuable resource and from any dominance–subordinance interactions weakens the competitive ability of males. After having been isolated from a group for 1 min, territorial males were mostly able to achieve territorial status upon return. After 15 min of isolation, however, the ability

of the territorial male to take back the territorial position became similar to that of subordinate males. Nevertheless, whether the mere experience of sitting on the host or the experience of winning dominance–subordinance interactions is more important in shaping the males' competitive ability needs further investigation.

The territorial system in *Nv* is less stable in larger than in smaller groups. Take-over events only happened in relatively large groups (eight and 10 males), replacement of the territorial male after removal was observed less often in larger (more than five males) than in smaller groups and samples in which two males shared the position on the host also consisted of more than five males. Van den Assem, Gijswijt, et al. (1980) mentioned that the territorial system in *Nv* breaks down at high male densities (see also van den Assem, 1986, 1996). With an increasing number of challengers, it gets harder for the territorial male to defend the territory over a prolonged time which is likely to result in frequent take-over events and finally scramble competition. In the dung fly *Scatophaga stercoraria*, males establish territories at oviposition sites when densities are low but switch to scramble competition at higher densities (Borgia, 1980). Similar effects have been suggested for and found in several other animal species (Alcock & O'Neill, 1986; Mills & Reynolds, 2003; Moore, 1987; Parker & Knowlton, 1980; Warner & Hoffman, 1980). Although the sex ratio in *Nv* is strongly female biased in the absence of competition, more balanced sex ratios may arise in nature when several females exploit host patches simultaneously (Werren, 1983). *Nv* are haplodiploid and can adjust offspring sex ratios during oviposition to account for different competitive pressures between males after emergence, a process called local mate competition (Hamilton, 1967; Werren, 1980). In a field study, Grillenberger, van de Zande, et al. (2009) found even strongly male-biased sex ratios. Hence, both situations are likely to occur in nature: low male densities with a territorial group structure and high male densities resulting in scramble competition.

Based on our observations and analysis, it was not possible to demonstrate priority of access to matings for territorial males. Females mated as often with subordinate as with territorial males and territorial males did not copulate more often than expected by chance. However, with our data, it was not possible to evaluate the mating success of single subordinate males. We rather compared the mating success of the territorial male with that of all subordinate males in the group taken together. It is thus possible that the mating success of single subordinate males is lower than that of territorial males and lower than expected by chance. In addition, the natural environment is usually more structured than the experimental arena we used. Courtship and mating by territorial males might therefore happen unnoticed

by other males or be noticed by only a few other males. Furthermore, the mating success of subordinate males may vary between individuals. In our recordings, aggressive interactions did not always involve territorial males but occurred also between pairs of subordinate males (see representative Movie S1 in the Supplementary material of the published article) and some subordinate males showed marking activity on the periphery of the arena. It is thus possible that the dominance–subordinance relationships in *Nv* male groups are even more complex than described so far.

Subordinate males in our study frequently exhibited a different mating strategy than territorial males. While all territorial males honestly courted females prior to copulation, half of the subordinate males achieved copulation by sneaking in when females opened their genital orifice. Sneaking behaviour in *Nv* has been described in earlier studies (van den Assem, 1996; van den Assem, Gijswijt, et al., 1980; van den Assem & Vernel, 1979) and is a common conditional mating strategy in animals (Gross, 1996).

Several females in our experiments mated with two or three males consecutively. The frequency of multiple mating in females of *Nv*, however, increases as a result of prolonged breeding in the laboratory (van den Assem & Jachmann, 1999; Burton-Chellew, Beukeboom, et al., 2007). In nature, multiple matings occur only rarely (Grillenberger et al., 2008). As we used a strain of *Nv* that has been kept in the laboratory for many years, we assume that multiple mating in this study arose from prolonged laboratory rearing rather than reflecting frequencies found in the field. The analysis of mating success was therefore restricted to the females' first mating partners.

Males of *Ng* exhibit a completely different behavioural strategy at the natal host patch than males of *Nv*. They readily disperse from the host right after emergence, court any female they encounter and, although they do not show territorial behaviour, they show an abdominal marking activity on the periphery which is similar to that of males of *Nv*. Females of *Ng* are almost invariably mated when they emerge from the host (Drapeau & Werren, 1999; Giesbers et al., 2013; Leonard & Boake, 2006). In addition, during our recordings *Ng* females emerged from the host prior to males. For males it thus makes sense not to stay near the host puparium after emergence. Nevertheless, five *Ng* females mated with males outside the host. Based on our experimental set-up we do not know, however, whether these females mated for the first time (i.e. they emerged unmated) or remated after having mated inside the host before emergence (e.g. as a result of prolonged laboratory rearing similar to the effect observed in *Nv*; van den Assem & Jachmann, 1999; Burton-Chellew, Beukeboom, et al., 2007).

Marking the substrate with a pheromone that is attractive to virgin females seems to be pointless when all females in the surroundings are already mated. However, marking activity could be very important for multiparasitism in microsympatry with *Nv*. Based on a study on within-host mating in the genus *Nasonia*, Giesbers, Pannebakker, van de Zande, & Beukeboom (2016) showed that males of *Ng* control female emergence from the host by refraining from chewing an exit hole into the host puparium. Under multiparasitism with *Nv* almost all *Ng* females emerged unmated after *Nv* males had opened the host. These virgin *Ng* females could be attracted to pheromonal markings applied to the substrate by *Ng* males. Microsympatry and multiparasitism are common among *Nv* and *Ng.* In fact, Grillenberger, van de Zande, et al. (2009) found both species within the same host in 29% of parasitised fly pupae. Furthermore, *Ng* was never found in the absence of *Nv*. This renders the pronounced marking activity of *Ng* males highly relevant in the natural environment.

In accordance with the behavioural differences exhibited at the natal host patch, *Nv* and *Ng* further differ in the temporal pattern of emergence and dispersal. *Nv* are protandrous and the succession of emergences is much slower than in *Ng*. As a result of positive selection on protandry in *Nv* (Moynihan & Shuker, 2011), the first males emerge from their hosts as soon as possible. In contrast, in *Ng* an exit hole is chewed when females are already mated and restless, resulting in a quick succession of emergences. After emergence, males of *Ng* dispersed to the other two compartments of the microcosm arena while none of the males of *Nv* left the emergence site. A similar observation has been made by Leonard and Boake (2006). For *Nv* it has been suggested that subordinate males may wander off and establish a territory on a host from which wasps would soon emerge (van den Assem, Gijswijt, et al., 1980; King et al., 1969). Males of *Nv* can discriminate between parasitised and non-parasitised hosts and are particularly attracted to hosts from which females are about to emerge (King et al., 1969; Shuker et al., 2005). In our experimental set-up, we presented an additional parasitised host (alternative site) as an opportunity for subordinate males to establish a territory when competition at the natal host patch increased. However, none of the *Nv* males left the emergence site. The distance between the natal host and the alternative host in the microcosm was relatively large compared to the distances between hosts in host choice experiments conducted by King et al. (1969) and the compartments in the microcosm arena were connected by narrow tunnels which may have prevented dispersion to the alternative site by *Nv* males. However, similar distances and barriers probably occur in naturally structured environments as well. In nature, *Nv* males are therefore unlikely to wander off to hosts that are not directly adjoined to the host from which they have emerged.

In conclusion, *Nv* and *Ng* exhibit highly different behavioural strategies at the natal host patch. Males of *Nv* do not disperse and either become territorial or, if they are subordinate, engage in sneaking behaviour to gain mating opportunities. The repeated aggressive interactions among subordinate males in our experiments indicate that the territorial system of *Nv* may be even more complex than described so far. As our experiments focused on the behaviour of the territorial male dominating the position on the host, we were not able to detect differences between subordinate individuals. In addition it would be interesting in future studies to investigate more complex experimental set-ups in which wasps emerging from two or more parasitised hosts are observed together in the same arena. Such experiments may give valuable insights into dynamics arising between several territory owners, a situation that probably occurs in the natural environment considering the clumped distribution of hosts. Males of *Ng*, on the other hand, predominantly mate inside the host, disperse after emergence and engage in courtship outside the host whenever they encounter a female. We hypothesise that the marking behaviour exhibited by males of *Ng*, although unnecessary when occurring alone, is adaptive in situations where the two species co-occur within the same host in microsympatry. Future investigations in the other two *Nasonia* species and studies investigating the adaptive value of the different aspects of the behavioural strategies under multiparasitism as well as the effect of microsympatry on offspring numbers in the different *Nasonia* species may help us understand the mechanisms leading to the evolution of diverging behavioural strategies in order to avoid interspecific reproductive interference among closely related species.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.anbehav.

Supplementary Movie S1 Two-minute-subsample taken from the microcosm video recordings of a *Nasonia vitripennis* male group at the emergence site. The territorial male defends the host in the middle of the arena against five male competitors. It spends more time on the host than each of the subordinate males and wins male-male contests more often. This subsample was used in the analysis of male territoriality, dominance-subordinance interactions and male marking behavior (territoriality plot shown in Fig. 1, column 3, row 3).

4. The chemical basis of mate recognition in two parasitoid wasp species of the genus *Nasonia*

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ABSTRACT

To recognise one's mate is essential for all sexually reproducing animals. In insects, mate recognition is often based on chemical cues such as hydrocarbons which are distributed over the insect's cuticle. In the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae), interspecific mating possibly occurs in microsympatry between *Nasonia vitripennis* Walker and *Nasonia giraulti* Darling despite post-zygotic isolation mechanisms preventing hybridization. Males of *N. vitripennis* are known to equally court con- and heterospecific females, which they recognise by means of cuticular hydrocarbons. A recent study surprisingly showed that this might not be the case in *N. giraulti*, leaving open how males of this species achieve the recognition of mating partners. In the present study, we investigated chemical mate recognition in *N. giraulti* in more detail and compared observed behaviours with behaviours of *N. vitripennis* by conducting experiments with both species concurrently and under the same experimental conditions. We disentangled the role of female-derived non-polar cuticular lipids – i.e., cuticular hydrocarbons – and more polar cuticular lipids in the ability of males to recognise con- and heterospecific females. In addition, we tested whether females of the two species discriminate similarly between con- and heterospecific males. We demonstrate that, in contrast to *N. vitripennis*, males of *N. giraulti* prefer live conspecific females over heterospecific ones. Furthermore, in contrast to *N. vitripennis*, mate recognition in *N. giraulti* males is not based on cuticular hydrocarbons, but rather involves other chemical messengers, presumably more polar cuticular lipids. In both species discrimination against heterospecific males decreases with female age.

INTRODUCTION

Correct mate recognition is essential for all sexually reproducing organisms. For successful reproduction, a male encountering another individual needs to decide whether it belongs to the same species and the opposite sex. Reliable identification of potential mates can save time and energy otherwise invested in courtship and/or sperm transferred to the wrong recipients (Gröning & Hochkirch, 2008). Similarly, females need to decide whether the courting male belongs to the same species to avoid fitness costs due to fertilization by heterospecific sperm leading to unviable or no offspring, infertile hybrids, or hybrid breakdown in subsequent generations (Orr & Presgraves, 2000; Shapiro, 2000; Geuverink et al., 2009). As females usually invest more energy in the production of offspring than males, which is associated with higher costs of heterospecific mating, mate discrimination is expected to be stronger in females than in males (Trivers, 1972). This is particularly important in species constellations in which females mate only once and post-mating isolation is complete (Liou & Price, 1994). Although females should be choosier than males, mate discrimination by either sex can act as an effective pre-mating reproductive isolation mechanism to avoid costs of heterospecific mating.

In insects, recognition processes are widely based on chemical cues or signals (Wyatt, 2014). Chemical stimuli perceived by direct contact in insects are the cuticular hydrocarbons (CHCs; Singer, 1998). Besides functioning as an effective barrier preventing insects from desiccation (Gibbs, 1998), CHCs evolved into serving a plethora of functions concerning communication between and within insect species (Blomquist & Bagnères, 2010). Information encoded by CHCs include species, sex, and mating status in various insects as well as group membership, age, dominance, and fertility status in social Hymenoptera (Howard, 1993; Singer, 1998; Howard & Blomquist, 2005; Blomquist & Bagnères, 2010). In parasitic wasps, CHCs have been shown to serve as contact sex pheromones, which allow males to address courtship to adequate mating partners (Syvertsen et al., 1995; Ruther et al., 2000; Sullivan, 2002; Ruther, Döring, et al., 2011; Ablard et al., 2012, Ruther, 2013).

In the parasitoid wasp *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae; 1836), female-derived CHCs elicit courtship behaviour in males, whereas male-derived CHCs do not (Steiner et al., 2006). Males of *N. vitripennis*, however, do not discriminate between con- and heterospecific females and engage equally in mounting and courtship activities with females of different *Nasonia* species (Giesbers et al., 2013; Buellesbach et al., 2014). Females of *N. vitripennis* on the other hand mate more often with con- than with

heterospecific males (Bordenstein & Werren, 1998; Giesbers et al., 2013; Buellesbach et al., 2014; Ruther et al., 2014). A possible mechanism for females to recognise conspecific males is the males' courtship behaviour, which consists of a stereotypic sequence of distinct behavioural elements. These include mounting the female and bouts of head-nodding behaviour – i.e., moving of the male mouthparts along the female's antennae – coupled with the transfer of a male aphrodisiac pheromone, eventually leading to female receptivity and copulation (van den Assem, Jachmann, et al., 1980; Ruther et al., 2010). After copulation, males perform post-copulatory courtship including additional series of head-nodding behaviour. A detailed analysis of the courtship behaviour in the four *Nasonia* species revealed some subtle species-specific differences (van den Assem & Vernel, 1979; van den Assem, Jachmann, et al., 1980; van den Assem et al., 1981; van den Assem & Werren, 1994). Although not completely strict, the females' choosiness has the potential of playing a major part in maintaining prezygotic reproductive isolation between sympatric *Nasonia* species. Choosiness is especially important for females, as they usually mate only once before switching to host-seeking behaviour (King et al., 2000; Grillenberger et al., 2008; Ruther et al., 2014).

The genus *Nasonia* comprises four species all developing on pupae of cyclorrhaphous flies (Whiting, 1967; Darling & Werren, 1990; Raychoudhury, Desjardins, et al., 2010). All four species are gregarious parasitoids, i.e., females lay more than one egg into a fly puparium. After hatching, larvae feed as ectoparasites on the fly pupa inside the fly puparium, and emerge from the host after eclosion as adults. Except for *Nasonia giraulti* Darling and *Nasonia oneida* Raychoudhury & Desjardins, the *Nasonia* species are reproductively isolated by cytoplasmic incompatibility resulting from infections with different strains of the intracellular bacterium *Wolbachia* (Bordenstein et al., 2001). As a consequence, eggs fertilised by heterospecific sperm develop into male offspring and no viable hybrids are produced (Breeuwer & Werren, 1990).

Nasonia vitripennis is cosmopolitan and frequently occurs in microsympatry, i.e., within the same host individual, with each of the other three species, including *N. giraulti* in the eastern part of North America (Darling & Werren, 1990; Grillenberger, van de Zande, et al., 2009; Raychoudhury, Desjardins, et al., 2010; Raychoudhury, Grillenberger, et al., 2010). In all *Nasonia* species, mating typically occurs at the natal host patch, from which mated females, but not males, disperse (King et al., 2000; Grillenberger et al., 2008; Ruther et al., 2014). Microsympatry, thus, possibly leads to interspecific mating. As a result, a clear discrimination between con- and heterospecific mating partners would be expected in all

Nasonia species to maintain species integrity and avoid costs imposed by interspecific mating.

In contrast to *N. vitripennis*, the chemical basis of mate recognition in the other three *Nasonia* species is not well understood yet. Two recent studies showed surprisingly that males of *N. giraulti*, confronted with dead females, preferred *N. vitripennis* females over conspecific ones (Buellesbach et al., 2013; Giesbers et al., 2013). The same preference was observed, when complete cuticular lipid extracts (hexane extracts) of female wasps were applied to washed wasp cadavers (so-called dummies; Buellesbach et al., 2013). These results pointed to a seemingly non-adaptive preference for heterospecific over conspecific mating partners in *N. giraulti* males, which is somewhat difficult to explain from an evolutionary perspective. The findings also raise questions relating to observations from other studies, where *N. giraulti* males readily courted and mated con- and heterospecific females equally (live females: Buellesbach et al., 2014; dead females: Ruther et al., 2014). A shift from chemical communication to other modes of communication, e.g., via behavioural, tactile, or acoustic cues, has been suggested for *N. giraulti* (Buellesbach et al., 2013). In contrast to *N. vitripennis* which mate on top of or in close vicinity to the host puparium after emergence, wasps of *N. giraulti* usually mate inside the host puparium prior to emergence, a behaviour termed within-host mating (WHM; Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013; Ruther et al., 2014). Buellesbach et al. (2013) referred to the high WHM rates of up to 100% observed in *N. giraulti*, and hypothesised that these might render chemical messengers to recognise conspecific females unnecessary. However, even if mating occurs inside the host puparium, *N. giraulti* males need to recognise females and, given the confined space and low light levels inside the puparium, the use of chemical messengers seems to be very likely. Therefore, the role of chemical stimuli in the process of mate recognition needs further clarification in this species.

Here, we investigate the chemical basis of mate recognition behaviour in *N. giraulti* in more detail and compare the behaviour of *N. giraulti* with that of *N. vitripennis* by conducting concurrent behavioural bioassays with both species. Previous studies on the chemical basis of mate recognition in *N. giraulti* did not differentiate clearly between complete cuticular lipid extracts and fractions of extracts gained from purification procedures (Buellesbach et al., 2013; Giesbers et al., 2013). Irrespective of the solvent used for extractions, complete cuticular lipid extracts may contain more polar lipids, such as free fatty acids, alcohols, glycerides, sterols, aldehydes, or ketones, in addition to the non-polar lipids, i.e., CHCs (Lockey, 1988; Buckner, 1993; Kühbandner & Ruther, 2015).

Furthermore, polar cuticular lipids have been shown to function as pheromones in various insects (Buckner, 1993; Yew et al., 2009; Kühbandner et al., 2012). In this study, we therefore differentiate between complete cuticular lipid extracts and the non-polar lipid fraction containing the CHCs. Moreover, we avoid possible effects of inbreeding resulting from long-term laboratory rearing and effects of antibiotics treatment by using two genetically diverse strains, namely NvHVRx and NgMix (van de Zande et al., 2014; Giesbers, 2016). In a stepwise reduction of information content available for recognition and discrimination, we performed a set of behavioural experiments in which males were confronted with live females, dead females, complete cuticular lipid extracts of females, or non-polar CHC fractions of these extracts alone. Differences in the cuticular lipid composition between females of the two strains were furthermore investigated by coupled gas chromatography-mass spectrometry (GC-MS). In addition, we tested female mate discrimination, taking into account possible age effects in female choosiness. Younger (0-day-old) females of *N. vitripennis* are known to exhibit stronger discrimination against heterospecific males than older (2-day-old) females (Ruther et al., 2014). This age effect has not been shown for *N. giraulti* females yet. By performing bioassays with both species concurrently and under the same experimental conditions, direct comparisons between the two species were possible.

We hypothesised that both *N. giraulti* and *N. vitripennis* males recognise conspecific females and that this recognition process is, at least partially, based on female-derived chemical stimuli. We furthermore predicted that, if males of *N. giraulti* discriminate between con- and heterospecific females, they prefer conspecific ones. For *N. vitripennis*, we predicted that males do not discriminate between con- and heterospecific females. Finally, we hypothesised that, similar to *N. vitripennis*, females of *N. giraulti* discriminate between con- and heterospecific males and that there is an age-effect in this choosiness in both species.

MATERIALS AND METHODS

Rearing conditions and preparation of wasps

All experiments were performed with the two outbred laboratory strains of *N. vitripennis* and *N. giraulti*, NvHVRx and NgMix (van de Zande et al., 2014). Wasps were reared on *Lucilia caesar* L. (Diptera: Calliphoridae) pupae at 25 °C and L16:D8 photoperiod.

In each generation, hosts were mixed 7 days after oviposition according to the rearing procedure described in van de Zande et al. (2014). To control for adult wasp age, wasp pupae were isolated from fly puparia 1-2 days before expected emergence. Single individuals were then kept in 1.5-ml microcentrifuge tubes and emergence was checked every morning. On emergence day, wasps were considered 0 days old. All experiments were done with 2-day-old females (or extracts thereof, except in experiment 1) and 0- to 4-day-old males. Assignment of wasps to treatments was randomised.

Experiment 1 – Behavioural observations of living couples

We tested whether males of *N. vitripennis* and *N. giraulti* discriminate between live con- and heterospecific females. In addition, we tested whether females discriminate between males of the two species when courted and whether there is an age effect in female choosiness. Males of *N. vitripennis* and *N. giraulti* were tested in single confrontations with 0- and 2-day-old females of both species presenting females singly and resulting in eight different treatments (n = 20 per treatment). For observations we used a mating arena consisting of a round hole (1 cm diameter) cut in a 3-mm-thick sheet of acrylic glass. The arena was put on a piece of filter paper and closed by a glass slip after introduction of a female and a male. Behaviours were recorded with a Sony Nex-5 camera connected to a stereo microscope (Schott, KL 2000 LED) at 10× magnification. Recording lasted until the end of post-copulatory courtship. Recording was stopped if mating did not occur within 5 min. Movies were analysed at half-speed using the video module of The Observer XT v.11.5 software (Noldus, Wageningen, The Netherlands). The time from first antennal contact to when the male mounted the female (1st contact to mounting), the time from mounting to the start of head-nodding behaviour (mounting to head-nodding), the duration of the copulation (duration of copulation), and the duration of post-copulatory courtship (post-copulatory courtship) were recorded. These behaviours were considered to be possible indicators of male mate discrimination which are not influenced by the behaviour of the female. In addition, we recorded the duration of head-nodding behaviour (duration head-nodding) and whether copulations occurred or not (copulations). These two parameters were considered to be indicators for female mate choice. A short duration of head-nodding was considered to represent increased female interest in the specific male because head-nodding is terminated by the female's receptivity signal (head lowering and opening of the genital pouch).

Experiment 2 – Response of males to dead females

Dead females were presented to males to test whether males are able to recognise and/or discriminate between females excluding effects of female behaviour, such as receptivity signaling or movements. Dead females were presented either separately in single confrontations, or two females were presented in simultaneous confrontations (one of each species). In addition, a negative control was set up with solvent-washed females presented to males in single confrontations to check for the involvement of chemical stimuli in mate recognition (eight treatments: males of both species tested in single confrontations with females of either species and control, plus males of both species tested in simultaneous confrontations; n = 20 per treatment). Two-day-old *N. vitripennis* and *N. giraulti* females were killed by freezing and kept at -20 °C until being used but no longer than 3 days. For the control, dead females from both species were pooled and washed continuously with dichloromethane (DCM) (Roth, Karlsruhe, Germany) for 6 h using a Soxhlet extractor. To ensure that all extractable chemicals had been removed from these females, they were extracted again for 10 min in 20 µl DCM per individual and extracts were analysed by GC-MS. After evaporation of the solvent residues, dead females were glued on pieces of filter paper (55 mm diameter) and presented to males in the middle of the mating arena. In simultaneous confrontations, two females, one from each species, were glued next to each other with their heads pointing into the same direction. Positions of the two species were exchanged after every replicate. Video recording and behavioural analysis was similar to experiment 1 and lasted for 5 min after the test male's first antennal contact with a dead female. Behaviours recorded were: the time the male spent mounted on the female (time mounted) and the number of copulation attempts (copulation attempts). This procedure was the same in all further experiments.

Experiment 3 – Response of males to female-derived complete cuticular lipid extracts

Complete cuticular lipid extracts of females (solved in DCM) were applied on dummies and presented to males in single confrontations to test whether males are able to recognise and/or discriminate between females based on chemical messengers only, excluding female-specific visual and tactile cues. As a control, pure DCM was applied (six treatments: males of both species tested with extracts of females of either species and control; n = 20 per treatment). To avoid possible effects of female-specific cuticular structures and visual cues on male responses, solvent-washed males were used as dummies in this and the following

experiment. For dummy preparation, males of both species were killed by freezing, pooled and washed 3× for 10 min in 20 µl DCM per individual. The absence of cuticular lipids was confirmed by GC-MS analysis. For extract preparation, 40-100 dead 2-day-old females were pooled and extracted for 10 min in 20 µl DCM per individual. Complete cuticular lipid extracts were then concentrated to a final concentration of two female equivalents per µl DCM. Single randomly chosen male dummies were glued to filter papers and 1 µl of the extract to be tested was applied to each dummy by means of a 5 µl microsyringe (Hamilton, Bonaduz, Switzerland). Extracts were carefully applied to the dummies in small steps to avoid leakage of the extract to the filter paper. Dummies were tested in the bioassays between 30 min and 4 h after extract application. Bioassays and data collection followed the procedures described for experiment 2.

Experiment 4 – Response of males to female-derived cuticular hydrocarbon fractions

Complete cuticular lipid extracts were fractionated and the non-polar CHC fractions were tested alone in single confrontations to test whether CHCs alone are sufficient for the recognition of and/or discrimination between females. By this procedure, more polar cuticular lipids such as cholesterol, triacylglycerides, and free fatty acids were excluded. Pure DCM was used as a control (six treatments, n = 20 each). To isolate the CHCs, complete cuticular lipid extracts of both species (representing 50-100 female equivalents) were dried under a stream of nitrogen and re-dissolved in 200 µl hexane (Roth). Extracts were then applied to 100 mg SiOH cartridges conditioned by rinsing with hexane, and CHCs were eluted by washing cartridges twice with 200 µl hexane. The absence of oxygenated lipids was confirmed by GC-MS analysis. Prior to use, the solvent was evaporated again and the CHCs were re-dissolved in DCM to a concentration of two female equivalents per µl. Dummy preparation, bioassays and data collection followed the procedures described for experiment 3.

Chemical composition of female-derived cuticular lipids

The composition of compounds in extracts from *N. vitripennis* and *N. giraulti* females was analysed by GC-MS. Two-day-old females of both species were killed by freezing. For each sample, two females were pooled and extracted for 10 min in 25 µl of DCM containing 10 ng μl^{-1} tetracosane (C24) as an internal standard (n = 10 per species). Extracts were analysed on a Shimadzu QP2010 Plus GC-MS system equipped with a BPX-5 capillary column (30 m × 0.25 mm, 0.25 µm film thickness; SGE, Milton Keynes, UK). Aliquots of 2

µl of each extract were injected splitless at 300 °C by means of a Shimadzu AOC 20i autosampler. Oven temperature started at 150 °C, increased by 3 °C per min and was held at 300 °C for 20 min. Helium was used as carrier gas at a linear velocity of 50 cm s^{-1}. The interface temperature was 300 °C. Substances were ionised by electron impact ionization (EI) at 70 eV. Ion source temperature was 200 °C. An n-alkane mixture (C_7-C_{40}) was analysed under the same conditions to determine the relative retention indices (RIs) (van den Dool & Kratz, 1963). Compounds were identified by means of comparison of their RIs with literature data (Steiner et al., 2006; Niehuis et al., 2010; Buellesbach et al., 2013) and by the analysis of diagnostic ions in the mass spectra.

Statistical analysis

Differences in the responses of males towards conspecific and heterospecific females from experiment 1 (living couples) were analysed by two-tailed Mann-Whitney U-tests. In the analysis of 'duration of copulation' and 'post-copulatory courtship', only samples with successful copulations were included. Data from experiment 1 on behaviours connected to female mate discrimination were first tested for differences in the response towards con- and heterospecific males, pooling data of bioassays with 0- and 2-day-old females. Subsequently, we tested for age effects within con- and heterospecific couples separately. Data on mating rates (number of females consenting to mating) were analysed by 2×2 χ^2 tests. Differences in the duration of head-nodding behaviour were analysed by Mann-Whitney U-tests including only data from samples with successful copulations. As we expected females to discriminate against heterospecific males, and as we expected discrimination to be stronger in older females than in younger ones, one-tailed tests were performed. The time males spent mounted on dead females in simultaneous confrontations (experiment 2) was analysed by Wilcoxon signed rank tests for paired samples. Data from single confrontations with dead females (experiment 2) and from experiments with dummies (experiments 3 and 4) were analysed by Kruskal-Wallis H-tests followed by pairwise comparisons using Bonferroni-corrected Mann-Whitney U-tests. Two-tailed tests were performed for comparisons between con- and heterospecific stimuli, and one-tailed tests were performed for comparisons with washed females and controls. All non-parametric tests were performed for *N. vitripennis* and *N. giraulti* separately.

To compare the responses between the two species and between stimuli with differing information contents (comparing experiments 2, 3, and 4), additionally generalised linear models (glms) were performed, because these allow the implementation of more complex

designs including interactions. For experiment 1, a separate model was calculated for each recorded behaviour. Mating frequencies were analysed by fitting a logistic regression model (binomial distribution with logit link function). All other behaviours from experiment 1 were analysed by fitting models based on the proportion of time spent exhibiting the respective behaviour, with logit link function and assuming pseudo-binomial error structure (Faraway, 2016). Species, type of pairing (con- or heterospecific), and – in the case of mating frequencies and 'duration head-nodding' – female age were included as fixed factors. The proportion of time spent mounted on dead females or dummies in experiments 2 (only single confrontations), 3, and 4 were analysed together by fitting a glm with logit link function and pseudo-binomial error structure, and the corresponding numbers of males showing copulation attempts were analysed by fitting a logistic regression model. Species, type of pairing, and information content (dead females, complete cuticular lipid extract, CHC fraction) were included as fixed factors. For each model, factors and interactions not contributing significantly to the fit were dropped by a stepwise reduction of the model using the drop1 function in R.

The composition of female-derived cuticular lipids was investigated by multivariate analysis of the relative amounts of single compounds (or compound mixes in unresolved peaks). Relative amounts of single peaks were calculated by relating single peak areas to the area of the peak representing the internal standard. Peaks which represented on average less than 0.05% of the total amount of compounds per sample were neglected. A data matrix containing the relative amounts of all peaks per sample was set up. Differences in the lipid composition between the two species were analysed by a type II PERMANOVA (permutational multivariate ANOVA; Anderson, 2001) with 9 999 permutations using Bray-Curtis dissimilarities as distance measure. In addition, a SIMPER (similarity percentage) analysis was performed to detect peaks essential for the observed differences. Total amounts of cuticular lipids (in ng per individual) were calculated based on the internal standard peak area representing 20 ng of substance, and compared between *N. vitripennis* and *N. giraulti* females by means of a Mann-Whitney U-test. Moreover, differences in relative amounts of summed n-alkanes, monomethyl-, dimethyl-, trimethyl- and tetramethylalkanes were assessed. For graphical display, a non-metric multidimensional scaling (NMDS) plot on Bray-Curtis dissimilarities was drawn.

All analyses were done in R v.3.2.0 (R Development Core Team, 2015). An α-level of 0.05 was chosen as significance level for statistical tests.

RESULTS

Experiment 1 – Behavioural observations of living couples

Both *N. vitripennis* and *N. giraulti* males courted con- and heterospecific live females. In all behaviours considered to be possible indicators of male choosiness, *N. vitripennis* males did not differentiate between females of the two species (Mann-Whitney U-test, 1st contact to mounting: P = 0.23; mounting to head-nodding: P = 0.30; duration of copulation: P = 0.82; post-copulatory courtship: P = 0.10; Figure 1A,C,E,G). *Nasonia giraulti* males took more time to mount females and to start head-nodding when exposed to heterospecific as compared to conspecific ones (Mann-Whitney U-test, 1st contact to mounting: P = 0.01; mounting to head-nodding: P = 0.04; Figure 1B,D). No significant differences were observed in the durations of copulation and post-copulatory courtship in *N. giraulti* (Mann-Whitney U-test, duration of copulation: P = 0.07; post-copulatory courtship: P = 0.09; Figure 1F,H). In general, *N. giraulti* males spent more time on post-copulatory courtship than *N. vitripennis* males (glm: $F_{1,111} = 87.3$, P<0.0001; Table S1).

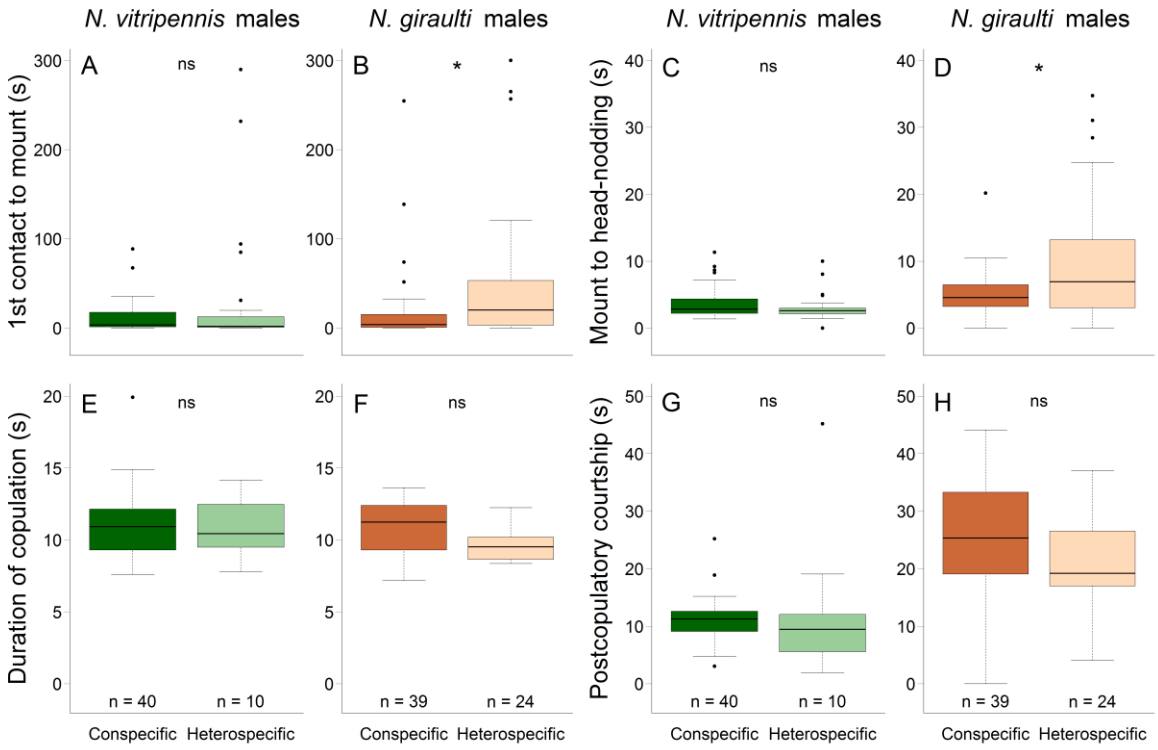

Figure 1 Box plots of behaviours associated with (A, C, E, G) *Nasonia vitripennis* and (B, D, F, H) *N. giraulti* male mate discrimination in bioassays with living con- and heterospecific couples. The behaviours are: (A, B) time between first antennal contact and mounting the female, (C, D) time between mounting the female and starting courtship (head-nodding behaviour; A-D: n = 20 each), (E, F) duration of copulation, and (G, H) duration of post-copulatory courtship (E-H: sample sizes are below the boxes). Boxes indicate median (horizontal line within the box), 25-75% quartiles (upper and lower box margins), and maximum/minimum

range (whiskers), and the dots indicate outliers (1.5× above box height). Asterisks indicate significant differences between con- and heterospecific couples (Mann-Whitney U-tests: P<0.05; ns, non-significant).

In both species, females discriminated between con- and heterospecific mating partners. In general, females mated more often with conspecifics than with heterospecifics (2×2 χ^2 test: *N. vitripennis*, $\chi^2 = 48$; *N. giraulti*, $\chi^2 = 16.8$, both P<0.0001; Figure 2A,B). Discrimination against heterospecific mates was stronger in *N. vitripennis* than in *N. giraulti* females (logistic regression model, % mated, species*type of pairing: $\chi^2 = 3.9$, P = 0.049; glm, head-nodding, species*type of pairing: $F_{1,108} = 4.6$, P = 0.03; Table S2). Prior to copulation, heterospecific males had to spend more time in head-nodding than conspecific males (Mann-Whitney U-test: *N. vitripennis*, P<0.0001; *N. giraulti*, P<0.01; Figure 2C,D).

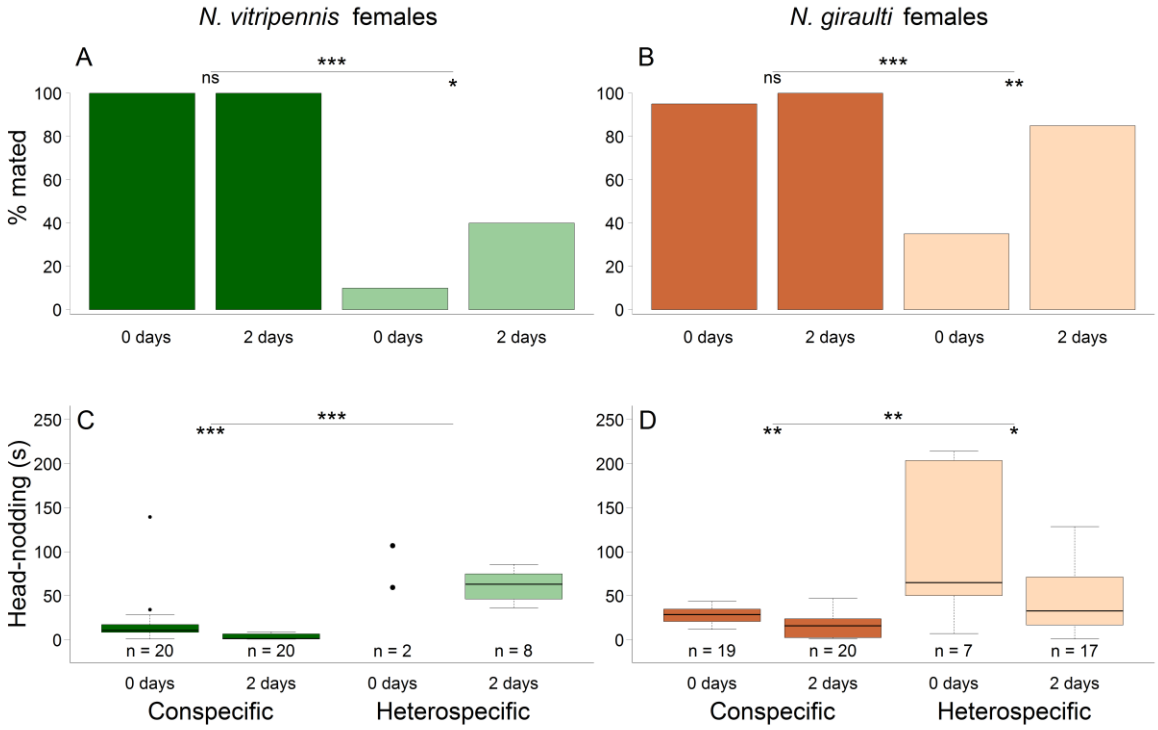

Figure 2 Behaviours associated with (A, C) *Nasonia vitripennis* and (B, D) *N. giraulti* female mate discrimination (displayed according to female age) in bioassays with living con- and heterospecific couples. The behaviours are: (A, B) percentage of females consenting to copulation (n = 20 each) and (C, D) duration of male courtship behaviour (head-nodding) before consenting to copulation (sample sizes are below the boxes). Boxes in panels C and D indicate median (horizontal line within the box), 25-75% quartiles (upper and lower box margins), maximum/minimum range (whiskers), and the dots indicate outliers (1.5× above box height). Asterisks indicate significant differences between con- and heterospecific couples (A, B, copulations: 2×2 χ^2 tests; C, D, head-nodding: Mann-Whitney U-tests: *0.01<P<0.05, **0.001<P<0.01, ***P<0.001; ns, non-significant).

In conspecific pairings, all females, except one 0-day-old *N. giraulti* female, became receptive. Two-day-old females became receptive faster than 0-day-old ones (Mann-Whitney U-test, head-nodding: *N. vitripennis*, P<0.001; *N. giraulti*, P<0.01). Also in heterospecific pairings, an age effect in female mate discrimination was found in both species, with 2-day-old females becoming receptive more often than 0-day-old ones (2 × 2 χ^2 test: *N. vitripennis*, $\chi^2 = 4.8$, P<0.05; *N. giraulti*, $\chi^2 = 10.42$, P<0.01). Due to the low number of females that consented to heterospecific mating in *N. vitripennis* (n = 2 and 8, for 0- and 2-day-old females, respectively), no statistical test was performed on the duration of head-nodding in this species. In *N. giraulti*, an age effect was found in the duration of head-nodding in heterospecific couples as well (Mann-Whitney U-test: P = 0.03).

Experiment 2 – Response of males to dead females

In simultaneous confrontations, males of both species spent similar amounts of time on con- and heterospecific dead females (Wilcoxon signed rank test: P>0.3 for both species; Figure 3A,B). Copulation attempts occurred in both species as often with con- as with heterospecific dead females (2 × 2 χ^2 test: *N. vitripennis*, $\chi^2 = 2.67$, P = 0.10; *N. giraulti*, $\chi^2 = 0.23$, P = 0.63). Also in single confrontations, males of both species did not discriminate between females of the two species but spent less time on solvent-washed dead females (Kruskal-Wallis test: P<0.0001; Bonferroni-corrected Mann-Whitney U-test pairwise comparisons: conspecific vs. heterospecific, P>0.3; conspecific vs. washed and heterospecific vs. washed, both P<0.0001; Figure 3C,D). Copulation attempts in single confrontations were observed as often with con- as with heterospecific dead females (2 × 2 χ^2 test: *N. vitripennis*, $\chi^2 = 2.11$, P = 0.15; *N. giraulti*, $\chi^2 = 0$, P = 1). Only one *N. vitripennis* male showed copulation attempts towards a washed dead female.

Experiment 3 – Response of males to female-derived complete cuticular lipid extracts

When exposed to complete cuticular lipid extracts, *N. vitripennis* males spent similar amounts of time on dummies treated with con- and heterospecific extracts, respectively, whereas the pure solvent was less attractive (Kruskal-Wallis test: P<0.0001; Bonferroni-corrected Mann-Whitney U-test pairwise comparisons: conspecific vs. heterospecific, P = 1; conspecific vs. control and heterospecific vs. control, both P<0.0001; Figure 3E). Copulation attempts were performed similarly often towards dummies treated with con- and heterospecific extracts (2 × 2 χ^2 test: $\chi^2 = 0.07$, P = 0.8), and no copulation attempts were

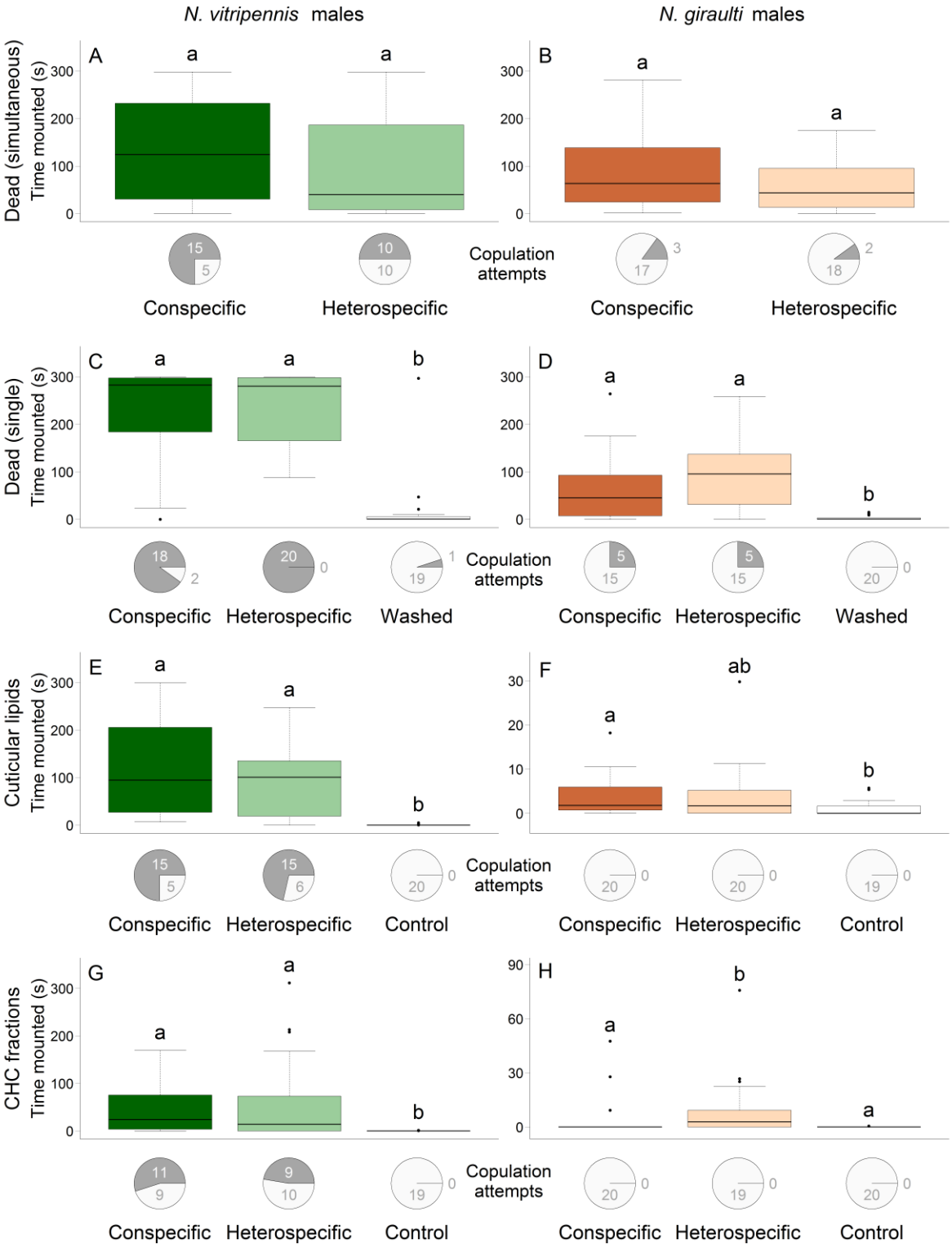

Figure 3 Box plots of the time (A, C, E, G) *Nasonia vitripennis* and (B, D, F, H) *N. girault* males spent mounted on dead females (A, B) presented together (simultaneous confrontations) or (C, D) presented separately (single confrontations), and on dummies applied with (E, F) complete cuticular lipid extracts of females, or (G, H) with cuticular hydrocarbon (CHC) fractions of female extracts (n = 20 each). Boxes indicate median (horizontal line within the box), 25-75% quartiles (upper and lower box margins), and maximum/minimum range (whiskers), and the dots indicate outliers (1.5× above box height). Washed: dead solvent-washed females; control: pure dichloromethane. Boxes within a panel capped with different letters indicate significant differences between treatment effects (Mann-Whitney U-tests: $P<0.05$). The pie charts indicate frequencies of samples with (grey) and without (white) copulation attempts.

observed towards the solvent control dummies. Likewise, *N. giraulti* males did not discriminate between female-derived extracts of the two species, spent more time on dummies applied with extract of conspecifics than on pure solvent, but did not discriminate between solvent control and extracts of heterospecific females (Kruskal-Wallis test: $P = 0.04$; Bonferroni-corrected Mann-Whitney U-test pairwise comparisons: conspecific vs. heterospecific, $P = 1$; conspecific vs. control, $P = 0.04$; heterospecific vs. control, $P = 0.18$; Figure 3F). No copulation attempts were performed by *N. giraulti* males in response to cuticular lipid extracts.

Experiment 4 – Response of males to female-derived cuticular hydrocarbon fractions

When exposed to dummies treated with the non-polar CHC fraction, *N. vitripennis* males spent more time on female CHCs than on the solvent control and did not differentiate between CHCs of the two species (Kruskal-Wallis test: $P<0.0001$; Bonferroni-corrected Mann-Whitney U-test pairwise comparisons: conspecific vs. heterospecific, $P = 1$; conspecific vs. control, $P<0.0001$; heterospecific vs. control, $P<0.001$; Figure 3G). Copulation attempts were performed equally often towards dummies applied with CHCs from con- and heterospecific females (2×2 χ^2 test: $\chi^2 = 0.2$, $P = 0.63$) and no copulation attempts were observed towards the solvent control. In contrast, *N. giraulti* males spent more time on CHCs of heterospecific females than on both CHCs of conspecific females and solvent control (Kruskal-Wallis test: $P<0.0001$; Bonferroni-corrected Mann-Whitney U-test pairwise comparisons: conspecific vs. heterospecific, $P = 0.02$; conspecific vs. control, $P = 0.83$; heterospecific vs. control, $P = 0.0001$; Figure 3H). No copulation attempts were performed by *N. giraulti* males in response to CHC fractions.

Analysis of the combined data from experiments 2-4 revealed significant differences in the general response of the two species. Males of *N. vitripennis* spent more time on dummies and attempted to copulate more often than males of *N. giraulti* (glm, time mounted: $F_{1,355} = 122.7$; logistic regression model, copulation attempts: $\chi^2 = 95.1$, both $P<0.0001$; Table S4). In addition, the response of both species decreased with lower information content available (from dead females over complete body extracts to the CHC fraction alone; glm, time mounted: $F_{1,352} = 173.8$; logistic regression model, copulation attempts: $\chi^2 = 39.4$, both $P<0.0001$), and this effect on copulation attempts was different between the two species (logistic regression model, species*information content: $\chi^2 = 6.1$, $P = 0.01$).

Chemical composition of female-derived cuticular lipids

Females of *N. vitripennis* and *N. giraulti* differed in the composition of their cuticular lipid profiles (PERMANOVA: Pseudo-$F_{1,18}$ = 26.8, P<0.001; Figure 4; see Table 1 for a list of all identified compounds). The differences did not result from differences in the amounts of single components but were rather based on the combined effects of many components taken together. Almost two-thirds of the analysed peaks were necessary to explain 90% of the observed differences between the two species and no single peak contributed more than 16.5% to these differences (SIMPER; Table 1). No differences were observed in the total amount of cuticular lipids between the species (*N. giraulti*: mean ± SD = 730 ± 210 ng, *N. vitripennis*: 680 ± 180 ng; Mann-Whitney U-test: P = 0.62). Nevertheless, differences were found in the branching patterns of CHCs. N-alkanes and monomethylalkanes were more abundant in *N. vitripennis* (Mann-Whitney U-test: P<0.0001 for both classes of alkanes), whereas multiply branched CHCs were more abundant in *N. giraulti* (Mann-Whitney U-test: dimethylalkanes: P<0.01; tri- and tetramethylalkanes: both P<0.0001). The only more polar lipid identified in the analysed extracts was cholesterol.

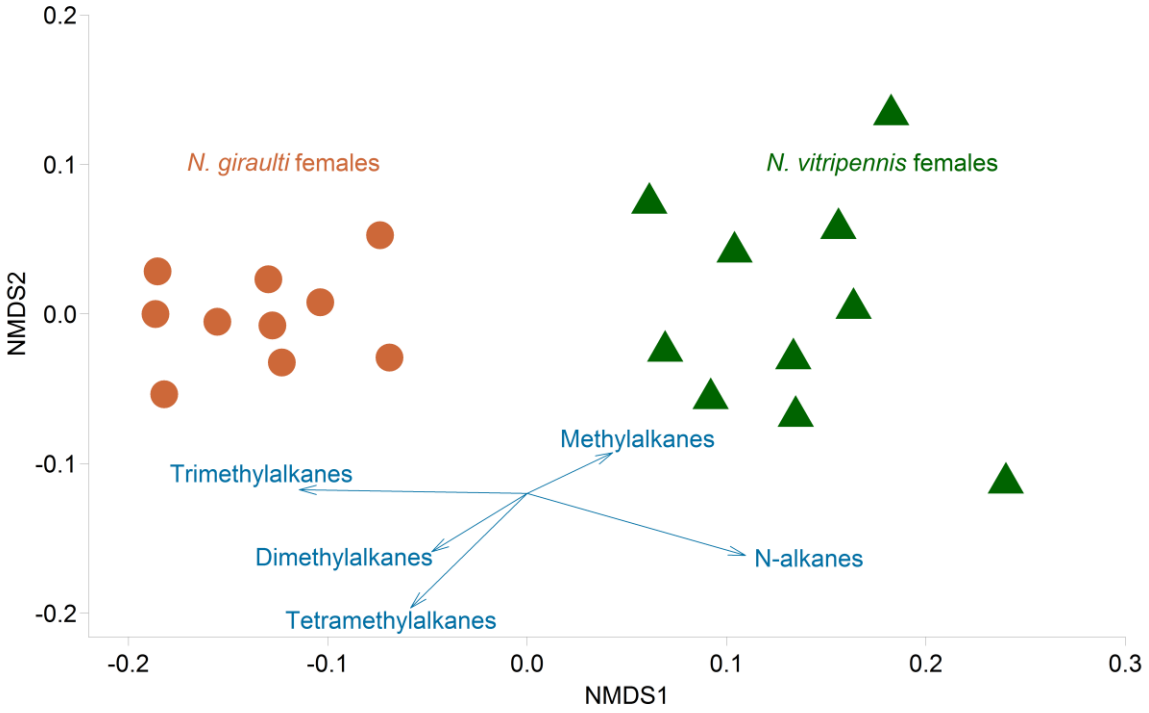

Figure 4 Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarities of relative amounts of chemical compounds found in dichloromethane (complete cuticular lipid) extracts of *Nasonia vitripennis* (triangles) and *N. giraulti* (circles) females. Each data point represents one sample. The distance between data points represents the degree of chemical dissimilarity between samples. The contribution of

various classes of hydrocarbons is represented by arrows, whose direction is based on the amount of the respective class, and whose length represents the intensity of the correlation.

DISCUSSION

Both, *N. vitripennis* and *N. giraulti* males readily courted and successfully mated with conspecific females. In contrast to Buellesbach et al. (2013), we were able to show that chemical messengers are involved in the recognition of females in both *N. vitripennis* and *N. giraulti.* In addition, in both species, the intensity of the response decreased with decreasing information content of the chemical stimuli applied. In *N. vitripennis*, CHCs were sufficient for the recognition of con- and heterospecific females. In contrast, *N. giraulti* males depended on complete cuticular lipid extracts to recognise conspecific females, the CHC fraction alone was not sufficient.

Possible candidates used in the recognition of conspecific females by *N. giraulti* males are more polar lipids (Buckner, 1993), such as alcohols, aldehydes, ketones, wax esters, and non-volatile fatty acid derivative (NFADs) such as triacylglycerides (TAGs). TAGs have been found to play an essential role in mate recognition in the parasitoid wasp *Lariophagus distinguendus* Förster (Kühbandner et al., 2012), and act as a brood pheromone in drone brood of the honey bee *Apis mellifera* L. (Koeniger & Veith, 1983). As they are not detected by standard GC-MS methods without transesterification into fatty acid methyl esters (Kühbandner & Ruther, 2015), NFADs were not detectable in the chemical analysis of cuticular lipids in this study.

Males of *N. vitripennis* recognise females by means of sex specific CHCs (Steiner et al., 2006). In accordance with the position of *N. vitripennis* at the basis of the phylogenetic tree (Campbell et al., 1993; Werren, 2010), recognition by CHCs seems to represent the ancestral state of mate recognition in this genus. If this is indeed the case, a shift to other chemical messengers must have happened in *N. giraulti*. Why this shift has happened remains unclear. Females of *N. giraulti* usually mate before emergence from the host, with reports of within-host mating rates ranging from 64 to 100% (Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013; Ruther et al., 2014). Due to the confined space inside the host, chemical stimuli might be generally less important in mate recognition in this species. In our study, *N. giraulti* males showed generally weaker responses to the chemical stimuli presented than males of *N. vitripennis*. Males of *N. giraulti* spent less time mounted on extract-coated dummies and never tried to copulate with them. Decreased stabilising

Table 1 Relative amounts (mean ± SD) of compounds found in complete cuticular lipid extracts (dichloromethane extracts) of females of *Nasonia giraulti* (strain NgMix) and *N. vitripennis* (strain NvHVRx)

Peak	RI	Compound names	Diagnostic ions	*N. giraulti*	*N. vitripennis*	Type	Contr. (%)
1	2900	C29	408	1.26 ± 0.74	2.29 ± 0.64	n	3.47
2	2939	7-MeC29	407(M-51), 112/113, 336/337	0.44 ± 0.36	0.97 ± 0.32	Mono	1.81
3	3000	C30	422	0.62 ± 0.05	0.93 ± 0.12	n	0.9
4	3100	C31	436	16.35 ± 1.81	22.17 ± 2.45	n	16.5
5	3128	Cholesterol	368, 386	6.46 ± 0.67	6.46 ± 0.94	Mono	2.55
		15-MeC31	435(M-15), 224/225, 252/253				
		13-MeC31	435(M-15), 196/197, 280/281				
		11-MeC31	435(M-15), 168/169, 308/309				
		9-MeC31	435(M-15), 140/141, 336/337				
6	3140	7-MeC31	435(M-15), 112/113, 364/365	5.19 ± 0.48	8.33 ± 2.05	Mono	8.88
7	3150	5-MeC31	435(M-15), 84/85, 392/393	5.34 ± 0.52	3.71 ± 0.71	Di	4.58
		13,x-DiMeC31	449(M-15), 196/197, 294/295				
		11,x-DiMeC31	449(M-15), 168/169, 322/323				
		9,x-DiMeC31	449(M-15), 140/141, 350/351				
8	3165	x,x-DiMeC31	449(M-15)	0.95 ± 0.13	0.86 ± 0.11	Di	0.44
9	3174	3-MeC31	421(M-15)	9.49 ± 0.91	9.08 ± 1.76	Mono	4.24
10	3202	3,x-DiMeC31	449(M-15), 435(M-29)	1.09 ± 0.08	0.87 ± 0.09	Di	0.68
		Unknown HC					
11	3228	3,x,x-TriMeC31	449(M-29, 435(M-43)	1.15 ± 0.15	0.96 ± 0.11	Tri	0.64
		5,x,x-TriMeC31	449(M-29), 435(M-43), 84/85				
12	3248	3,7,11,15-TetraMeC31	477(M-15), 463(M-29), 126/127, 392/393, 196/197, 322/323, 266/267, 252/253	0.96 ± 0.19	1.03 ± 0.2	Tetra	0.6
13	3300	C33	464	1.1 ± 0.21	1.43 ± 0.27	n	1.11
		Unknown HC					
14	3326	17-MeC33	463(M-29), 252/253	11.66 ± 0.93	10.63 ± 1.46	Mono	4.44
		15-MeC33	463(M-15), 225/225, 280/281				
		13-MeC33	463(M-15), 196/197, 308/309				
		11-MeC33	463(M-15), 168/169, 337/338				
15	3338	7-MeC33	463(M-15), 112/113, 392/393	1.11 ± 0.2	1.53 ± 0.17	Mono	1.23
16	3352	5-MeC33	463(M-15), 84/85, 420/421	4.11 ± 0.67	4.11 ± 0.67	Di	5.33
		15,x-DiMeC33	477(M-15), 224/225, 294/295				
		11,x-DiMeC33	477(M-15), 168/169, 350/351				
17	3362	7,23-DiMeC33	477(M-15), 112/113, 406/407, 168/169, 351/352	0.79 ± 0.1	0.25 ± 0.14	Di	1.49
		7,19-DiMeC33	477(M-15), 112/113, 406/407, 224/225, 294/295				
18	3372	x,15,x-TriMeC33	491(M-15), 168/169, 364/365	6.79 ± 0.65	2.55 ± 0.54	Tri	11.92
		Unknown HC					

Table 1. Continued

Peak	RI	Compound names	Diagnostic ions	*N. giraulti*	*N. vitripennis*	Type	Contr. (%)
19	3398	3,x-DiMeC33	477(M-15), 463(M-29)	1.49 ± 0.2	1.22 ± 0.23	Di	1
		Unknown HC					
20	3427	5,9,21-TriMeC33	491(M-15), 477(M-29), 84/85, 449/450, 154/155, 378/379, 350/351, 196/197	1.26 ± 0.14	0.86 ± 0.17	Tri	1.16
		x,7,x-TriMeC33	491(M-15), 477(M-29), 126/127, 406/407				
21	3446	3,7,11,15-TetraMeC33	505(M-15), 491(M-29), 126/127, 420/421, 196/197, 350/351, 266/267, 280/281	4.51 ± 0.48	1.31 ± 0.28	Tetra	9.04
22	3465	Unknown HC		0.32 ± 0.1	0.07 ± 0.07	-	0.74
23	3524	17-MeC35	491(M-15), 252/253, 280/281	4.62 ± 0.45	6.64 ± 0.76	Mono	5.66
		15-MeC35	491(M-15), 224/225, 308/309				
		13-MeC35	491(M-15), 196/197, 336/337				
24	3544	15,x-DiMeC35	505(M-15), 224/225, 322/323	5.54 ± 0.51	4.65 ± 1.12	Di	3.24
		13,x-DiMeC35	505(M-15), 196/197, 350/351				
		11,x-DiMeC35	505(M-15), 168/169, 378/379				
25	3560	7,23-DiMeC35	505(M-15), 112/113, 435/436, 280/281, 238/239	0.54 ± 0.12	1.42 ± 0.29	Di	2.54
		7,19-DiMeC35	505(M-15), 112/113, 435/436, 252/253, 294/295				
		7,15-DiMeC35	505(M-15), 112/113, 435/436, 196/197, 350/351				
26	3568	5,x-DiMeC35	505(M-15), 84/85, 462/463	3.25 ± 0.4	3.09 ± 0.41	Di	1.3
27	3596	3,15-DiMeC35	505(M-15), 491(M-29), 238/239, 308/309	0.28 ± 0.05	0.25 ± 0.12	Di	0.25
		Unknown HC					
28	3643	Unknown HC		0.45 ± 0.11	0.28 ± 0.12	-	0.54
29	3722	17-MeC37	519(M-15), 252/253, 322/323	0.64 ± 0.18	0.7 ± 0.26	Mono	0.7
		15-MeC37	519(M-15), 224/225, 350/351				
		13-MeC37	519(M-15), 196/197, 378/379				
30	3741	15,x-DiMeC37	533(M-15), 224/225, 322/323	1.07 ± 0.24	1.31 ± 0.2	Di	0.91
		13,x-DiMeC37	533(M-15), 196/197, 350/351				
		11,x-DiMeC37	533(M-15), 168/169, 406/407				
31	3758	7,x-DiMeC37	533(M-15), 112/113, 462/463	0.05 ± 0.08	0.05 ± 0.08	Di	0.83
32	3766	5,x-DiMeC37	533(M-15), 84/85, 490/491	1.09 ± 0.25	0.63 ± 0.18	Di	1.28

RI: retention index; type: assignment of peaks to hydrocarbon types for statistical analysis: n: n-alkane, Mono: monomethylalkane, Di: dimethylalkane, Tri: trimethylalkane, Tetra: tetramethylalkane. Contr.: contribution (%) to observed dissimilarities between the two species (SIMPER)

selection on the fidelity of the chemical signal could thus have led to a drift in the composition of CHCs in *N. giraulti* females. Irrespective of the intensity of the response, males of *N. giraulti* did not discriminate between con- and heterospecific dead female dummies as well as between con- and heterospecific whole body extracts. These results are in accordance with Ruther et al. (2014), but contradict results from Giesbers et al. (2013), where males showed a preference for *N. vitripennis* female dummies over conspecific ones. This is all the more surprising, as both of these studies were based on the same inbred strain, namely NGVA 2. However, differences in data acquisition and long-time laboratory rearing of separated populations might account for the different results. Nevertheless, in bioassays with living couples we showed, that *N. giraulti* males engage in courtship faster when the presented female is conspecific, indicating that they are able to discriminate between con- and heterospecific mating partners and that they prefer conspecific ones. It seems therefore that *N. giraulti* males use other modes of communication, e.g., tactile cues or female movements, in addition to chemical messengers to recognise and discriminate between females. In contrast to our study, Buellesbach et al. (2014) found no discrimination of *N. giraulti* males against *N. vitripennis* females. However, they investigated only male mate rejection rates, possibly overlooking more subtle indicators of mate discrimination such as time effects in courtship and copulation.

In contrast to *N. giraulti*, males of *N. vitripennis* did not discriminate between con- and heterospecific females, and they readily responded to the CHC fraction of con- as well as heterospecific female extracts. These results are in accordance with earlier studies (Buellesbach et al., 2014; Giesbers et al., 2013). As males of *N. vitripennis* showed generally stronger responses to chemical stimuli, are faster in mounting and starting courtship (van den Assem & Werren, 1994; van den Assem & Beukeboom, 2004), and are more aggressive concerning other behaviours linked to mating (aggressive male-male interactions: Leonard & Boake, 2006; sneaking behaviour: van den Assem & Beukeboom, 2004; Giesbers et al., 2013), they might be in general less sensitive to small changes in the composition of CHCs on the females' cuticle. Bioassays investigating the response of *N. vitripennis* males towards experimentally manipulated CHCs with decreased or increased relative amounts of individual substances could give valuable insights here (Kühbandner et al., 2013).

Nevertheless, the question remains why *N. giraulti* males recognise the CHC fraction of *N. vitripennis* female extracts. If there has indeed been a shift in the composition of CHCs in *N. giraulti* females, one possibility is that the response towards CHCs of *N. vitripennis* merely persisted when the CHC composition in *N. giraulti* females changed. Another

possibility is that *N. giraulti* males gain fitness benefits from engaging in courtship and copulation with heterospecific females. After emergence, males of *N. vitripennis* mark the substrate with a pheromone which is highly attractive to females (Ruther et al., 2007, 2008). During courtship, another pheromone is applied to the female antennae, leading to a behavioural switch as a result of which females cease to react to the abdominal sex pheromone (Ruther et al., 2010; Ruther & Hammerl, 2014). This switch is also triggered when the female is courted by a male of *N. giraulti* (Ruther et al., 2014). In addition, females of *Nasonia* usually consent to mating only once during their life time (Grillenberger et al., 2008). Females of *N. vitripennis* that have mated a heterospecific male are therefore likely to refuse mating again and might leave the host patch before having been inseminated by a conspecific. Due to cytoplasmic incompatibility, these females would then be unable to produce female offspring (Bordenstein et al., 2003). Clearly, an *N. giraulti* male having prevented an *N. vitripennis* female from mating a conspecific would only gain fitness benefits, if the probability that this female is going to oviposit on the same host patch as a conspecific female inseminated by the same male is considerably high. However, before such a scenario can be claimed, the proposed fitness benefits need to be demonstrated under field conditions.

Like *N. vitripennis*, females of *N. giraulti* discriminated against heterospecific males. In addition, we confirm that in *N. vitripennis* older females are less strict than younger females, and show that this age effect is also present in *N. giraulti* females. Decreased choosiness in older females is probably responsible for the high heterospecific mating rates in *N. giraulti* found in Giesbers et al. (2013). However, in Buellesbach (2014) 2- to 3-day-old *N. giraulti* females discriminated significantly against *N. vitripennis* males suggesting strain-related variability of this feature. Nevertheless, our results show that female age has the potential to change results from mating trials profoundly, and is likely to be of importance in the other two species of *Nasonia* as well.

In conclusion, female-derived chemical stimuli are used by males of both species to recognise mating partners, although to a different degree. Our results stress the importance to control for age in mating trials with *Nasonia* wasps. In addition, they emphasise that a broad range of chemical substance classes need to be considered when investigating chemical communication in insects, even if the investigated species are closely related.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Table S1 Results of the generalized linear model (with logit link function and pseudo-binomial error structure) calculated on the proportion of time males of *Nasonia vitripennis* and *N. giraulti* spent on post-copulatory courtship in bioassays with live con- and heterospecific couples. Species (*N. vitripennis*, *N. giraulti*) and type of pairing (conspecific, heterospecific) were included in the model. Factors and interactions not contributing significantly to the fit of the model were dropped using the drop1 function in R. Analysis was done in R v.3.2.0 (R Development Core Team, 2015).

	d.f.	Deviance	Residual d.f.	Residual Deviance	F	P
Null			112	735.61		
Species	1	326.68	111	408.94	87.332	1.2e-15

Table S2 Results of (A) the logistic regression model calculated on the number of copulations of *Nasonia vitripennis* and *N. giraulti* females, and (B) the glm (with logit link function and pseudo-binomial error structure) calculated on the proportion of time males had to spend on head-nodding behavior prior to copulation, in bioassays with live con- and heterospecific couples. Species (*N. vitripennis*, *N. giraulti*), type of pairing (conspecific, heterospecific), and age (0-, 2-days-old) were included in the model. Factors and interactions not contributing significantly to the fit of the model were dropped by a stepwise reduction of the model using the drop1 function in R. Analysis was done in R v.3.2.0 (R Development Core Team, 2015).

		d.f.	Deviance (χ^2)	Residual d.f.	Residual Deviance		P
A	Null			159	193.752		
	Species	1	5.142	158	188.610		0.023
	Type of pairing	1	77.164	157	111.447		<2e-16
	Age	1	16.432	156	95.015		5.0e-05
	Species*type of pairing	1	3.885	155	91.130		0.049
		d.f.	Deviance	Residual d.f.	Residual Deviance	F	P
B	Null			112	5011.4		
	Species	1	237.52	111	4773.8	8.9047	0.0035
	Type of pairing	1	1537.89	110	3236.0	57.6554	1.2e-11
	Age	1	669.68	109	2566.3	25.1064	2.1e-06
	Species*type of pairing	1	122.91	108	2443.4	4.6079	0.034

Table S3 Results of the generalized linear models calculated on the combined data from experiments 2-4 (see Material and methods section for description), based on separate analysis of (A) the proportion of time males of *Nasonia vitripennis* and *N. giraulti* spent mounted on dummies (glm with logit link function and pseudo-binomial error structure) and (B) the number of copulation attempts (logistic regression model). Species (*N. vitripennis*, *N. giraulti*), type of pairing (conspecific, heterospecific, solvent control), and information content (exp. 2: dead females (single confrontations); exp. 3: cuticular lipids; exp. 4: cuticular hydrocarbons fractions) were included as factors. Factors and interactions not contributing significantly to the fit of the model were dropped by a stepwise reduction of the model using the drop1 function in R. Analysis was done in R v.3.2.0 (R Development Core Team, 2015).

		d.f.	Deviance	Residual d.f.	Residual Deviance	F	P
A	Null			356	64626		
	Species	1	10080	355	54546	122.673	<2e-16
	Type of pairing	2	15038	353	39508	91.507	<2e-16
	Information content	1	14279	352	25229	173.776	<2e-16
		d.f.	Deviance (χ^2)	Residual d.f.	Residual Deviance		P
B	Null			356	421.54		
	Species	1	95.136	355	326.41		<2e-16
	Type of pairing	2	105.918	353	220.49		<2e-16
	Information content	1	39.367	352	181.12		3.5e-10
	Species*information content	1	6.096	351	175.03		0.014

5. Previous interspecific courtship impairs female receptivity to conspecifics in the parasitoid wasp *Nasonia longicornis* but not in *N. vitripennis*

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ABSTRACT

Interspecific sexual interactions are not uncommon in animals. In sympatry, females often face the risk of accidentally mating with a heterospecific male. Based on the actual risks imposed by the environment at a given time and place, females should be able to adjust their mate acceptance in order to avoid interspecific copulations as well as accidentally refusing to mate with a conspecific. We investigate the ability of females of the two parasitoid wasp species *Nasonia vitripennis* (*Nv*) and *N. longicornis* (*Nl*) to adjust their mate acceptance in response to previous unsuccessful courtship by heterospecific males. We show that *Nl* females are more reluctant to mate with a conspecific male when having been courted previously by a heterospecific male, but *Nv* females are not. We argue that this strategy is reasonable for *Nl* females but not for *Nv* females, which follow a different strategy to avoid the fitness costs imposed by heterospecific copulations.

INTRODUCTION

Reproductive interference is common among a wide variety of animal taxa (Gröning & Hochkirch, 2008). Due to incomplete species recognition during mate acquisition, signals sometimes attract individuals of the wrong species (Doherty & Howard, 1996; Groot et al., 2010), courtship is directed towards the wrong mating partners (Andrews et al., 1982), males try to copulate with heterospecific females (Singer, 1990), and females occasionally become receptive to heterospecific males (Takafuji et al., 1997). To avoid the fitness costs arising from interspecific copulations, the ability to discriminate between conspecific and heterospecific courtship partners usually evolves in the choosing sex (usually females; Wirtz, 1999). During mate acquisition, a female assesses the species of the courting male, and refrains from copulation if the male does not belong to the same species. Mate discrimination acts as an important prezygotic hybridisation barrier (Noor, 1999; Doi et al., 2001), and is particularly important in species in which females mate only once during their lifetime and post-mating reproductive isolation is complete (Liou & Price, 1994). In insects, mate discrimination usually involves chemical messengers (Wyatt, 2014), but it frequently includes additional signals, e.g., specific courtship displays, acoustic signals, or wing vibration patterns (Talyn & Dowse, 2004). Closely related species often resemble each other in their courtship display, and mate discrimination is not absolutely accurate (Chaplin, 1973; Boake et al., 2000; Gray, 2005). Females face both the risk of accidentally mating a heterospecific male and the risk of accidentally rejecting a conspecific. A trade-off thus arises between either becoming more selective in order to prevent interspecific copulations or broaden the range of stimuli that elicit receptivity in order to avoid accidentally rejecting a conspecific. Females should minimise both of these risks by adjusting their behaviour depending on the actual risks and costs imposed by the environment at a given time and place. If the chance of meeting and being courted by a heterospecific partner is low, e.g., in areas of allopatry, the costs of rejecting a conspecific become comparably large, and the range of partners accepted for copulation should be broadened. In contrast, in sympatry, the chance of being courted by a heterospecific male increases with the increasing population density of the interfering species, and

interspecific copulations become more likely. In these situations, it is advantageous for females to become more selective by either establishing more accurate discrimination abilities or accepting the accidental rejection of a conspecific in order to avoid the much higher costs of consenting to interspecific copulation. It has been shown in various animal taxa that when animals are reared in sympatry with an interfering species, mate discrimination abilities can become more accurate through learning by experience either early in their life (e.g., sexual imprinting) or at later stages of their lives (e.g., contextual behavioural plasticity; Irwin & Price, 1999; Kozak & Boughman, 2009; Crowder et al., 2010). One means by which females may learn about the risks of interspecific mating in a specific environment is the experience of being courted by a heterospecific male. However, nothing is known to date about the direct effects of heterospecific courtship experience on future female mate acceptance.

Nasonia vitripennis (*Nv*) and *N. longicornis* (*Nl*) are two species of parasitoid wasps that parasitise the pupae of cyclorrhaphous flies. In the western part of North America, *Nv* and *Nl* occur frequently in microsympatry, i.e., they develop within the same host individual (Whiting, 1967; Darling & Werren, 1990; Grillenberger, van de Zande, et al., 2009; Raychoudhury, Grillenberger, et al., 2010). As fly pupae often occur in a clumped distribution, and *Nasonia* wasps are gregarious, i.e., they lay more than one egg per host individual, males and females of *Nv* and *Nl* likely encounter after emergence at the same host patch. Courtship and copulation in *Nasonia* happen typically at the natal host patch, and females leave after mating to search for new oviposition sites (King et al., 2000; Grillenberger et al., 2008; Ruther et al., 2014). In laboratory studies, males readily engage in courtship with females of the other species (Giesbers et al., 2013; Buellesbach et al., 2014; Ruther et al., 2014; Mair et al., 2017). Males exhibit an elaborate courtship display, including specific movements of their forelegs over the female's head accompanied by series of head-nodding movements during which a sex pheromone is transferred from the male's oral glands to the female's antennae (van den Assem, Jachmann, et al., 1980; Ruther et al., 2010). The female shows receptivity by lowering her antennae and opening the genital orifice, and copulation follows. Although following the same general pattern, male courtship displays differ in detail between the different *Nasonia* species

(van den Assem & Vernel, 1979; van den Assem, Jachmann, et al., 1980; van den Assem et al., 1981; van den Assem & Werren, 1994). Females are able to discriminate between conspecific and heterospecific mating partners, but mistakes in mate discrimination occur (Bordenstein & Werren, 1998; Giesbers et al., 2013; Buellesbach et al., 2014; Ruther et al., 2014; Mair et al., 2017). In behavioural bioassays, *Nv* females usually discriminate more strongly against *Nl* males (more than 70% rejection of heterospecific males) than vice versa (less than 40% rejection of heterospecific males), and mate discrimination differs among different *Nasonia* strains (Giesbers et al., 2013; Buellesbach et al., 2014). However, differences in mate discrimination among *Nv* strains seem to be independent from the origin of these strains from areas of sympatry or allopatry with other *Nasonia* species (Giesbers et al., 2013). *Nv* and *Nl* show complete postzygotic reproductive isolation due to *Wolbachia*-mediated cytoplasmic incompatibility, preventing the production of hybrid females (Bordenstein et al., 2001). Similar to most Hymenoptera, *Nasonia* are haplodiploid, and eggs fertilised by heterospecific sperm either die or develop into male offspring, similar to unfertilised eggs (Bordenstein et al., 2001; Tram et al., 2006). In addition, *Nasonia* females mate only once during their lifetime in nature (Grillenberger et al., 2008). Females having copulated with a conspecific male usually refrain from mating again (van den Assem & Visser, 1976). Females consenting to interspecific copulation thus face particularly high fitness costs (Liou & Price, 1994). *Nv* females counteract these costs by increased remating with a conspecific male after having copulated with a heterospecific (Ruther et al., 2014). However, this effect has not been shown for *Nl*. Considering the fitness costs imposed on the females of the less discriminating species, *Nl*, it would be advantageous for them to adjust their mate acceptance behaviour depending on the actual presence or absence of heterospecific males.

Here, we investigate the impact of previous unsuccessful heterospecific courtship on the females' acceptance of conspecific mates in *Nv* and *Nl*. We hypothesize that females of *Nl* use the experience of heterospecific courtship to adjust their mate acceptance behaviour. In particular, we hypothesize that *Nl* females that have been unsuccessfully courted by a heterospecific male are subsequently more reluctant to mate in general in order to avoid

accidentally copulating with the wrong male. We expect this reluctance to be reflected in both a decrease in conspecific mating rate and an increase of the duration of courtship necessary to induce receptivity in couples where copulation happens. For *Nv* females, we hypothesize that if they adjust their mating rates, they do so to a lesser degree because, firstly, *Nv* females show stronger mate discrimination in general, and secondly, they counteract costs of heterospecific matings by increased remating with a conspecific. We address these hypotheses by performing mating trials with *Nv* and *Nl* females without prior contact to any male, and with females that have been courted previously by a heterospecific male.

We found that *Nl* females, but not those of *Nv*, decreased conspecific mate acceptance after having been courted by a heterospecific male. We argue that this strategy is advantageous for *Nl* females to avoid future mismating and that the difference in behavioural plasticity between the two *Nasonia* species is reasonable, considering that *Nv* females, as shown in earlier studies, follow a different behavioural strategy to counteract the costs imposed by interspecific copulation through increased remating.

MATERIALS AND METHODS

Strains, Rearing, and Preparation of Wasps

Experiments were performed with the *Nv* strain NvHVRx (van de Zande et al., 2014) and the *Nl* strain NLMN8510*. Wasps were reared on freeze-killed pupae of the green bottle fly *Lucilia caesar* at 25 °C under a 16:8 light:dark regime. For behavioural bioassays, wasps were isolated from host puparia at their pupal stage, separated, and kept singly in 1.5-mL microcentrifuge tubes until being used in experiments. By isolating wasps at this developmental stage, it was ensured that adult wasps were unmated and had not had any direct contact with other adult wasps when the experiments began. Each day in the morning, isolated wasps were checked for emergences. Emerged wasps were sexed and defined as zero days old. For bioassays, zero-day-old females and one to three-day-old males were used.

Behavioural Bioassays

Mating trials were conducted in a standard mating arena consisting of a round hole (10 mm diameter, 3 mm height) cut into an acrylic glass plate and covered by a cover slip (for a detailed description of the arena, see Ruther et al., 2000). In each mating trial, a female and subsequently a male were put into the arena. The arena was closed with a cover slip, and the couple was observed for five minutes. The females of each species were subjected to one of two treatments: (1) Females were tested with conspecific males without prior contact to any other individual. (2) Females were exposed to a heterospecific male for five minutes, and were eventually courted by the male. If the female did not show receptivity to the heterospecific male, the heterospecific male was removed, and the female was subsequently exposed in a second mating trial to a conspecific male. For each conspecific couple, it was noted whether the female consented to mating or not. If copulation happened, the duration of preceding courtship, i.e., the time span between the male mounting the female and the female's receptivity signal (opening the genital orifice), was recorded.

Trials in which males (conspecific or heterospecific) did not engage in courtship were discarded. Trials in which the female showed receptivity to the heterospecific male were excluded from further treatment, but were noted in order to assess whether the exclusion of these females led to a bias in the experimental females. Excluding females that consented to interspecific mating could have potentially resulted in testing only those females in treatment two that were in general more reluctant to mate. However, only three *Nv* females (3.6%) and four females of *Nl* (4.8%) copulated with heterospecific males. Thus, it is unlikely that excluding these females resulted in a significant bias among the experimental groups.

Some *Nv* males exhibited aggressive behaviours towards *Nl* females. For each trial with *Nl* females, it was therefore noted whether the female was treated aggressively or not. Females were defined as having been treated aggressively when males, after having started courtship, turned their wings into a vertical position, jumped towards the female, and repeatedly grabbed the female, occasionally involving injuries, i.e., tearing off parts of the female's legs or

antennae. In these occasions, females usually crouched down and tried to run away from the respective males.

Each individual was tested only once. The assignment of individuals to treatments was randomised, and the order of treatments followed a blocked design. In total, 80 replicates were performed for each of the four treatments (*Nv* courted previously, *Nv* without prior contact, *Nl* courted previously, and *Nl* without prior contact).

Statistical Analysis

Conspecific mate rejection rates were compared between females having been courted previously and those without prior contact to heterospecific males using a 2 × 2 Chi-square test for each of the species separately. Differences in the duration of courtship were compared between females having been courted previously and those without prior contact to heterospecific males with a Mann–Whitney U test. In addition, conspecific mate rejection rates and the duration of courtship were compared between *Nl* females that had been subjected to aggressive versus non-aggressive heterospecific contact with a 2 × 2 Chi-square test and a Mann–Whitney U test, respectively.

RESULTS

Conspecific Mate Rejection

Nl females that have been courted by *Nv* males rejected conspecific males more often than females without prior heterospecific contact (Chi-square test: $\chi_1 = 9.56$, $n = 80$ each, $p < 0.01$; Figure 1A; all raw data are provided in Table S1 in the online supplementary material of the published article). In *Nv* females, heterospecific courtship had no effect on subsequent conspecific mate rejection (Chi-square test: $\chi_1 = 0.28$, $n = 80$ each, $p = 0.60$: Figure 1B).

Duration of Courtship

Previously courted *Nl* females that subsequently consented to mating a conspecific male did so after prolonged courtship compared to females without prior heterospecific contact (Mann–Whitney U test: U = 2206.5, $n = 53$ (*Nl* courted) and 61 (*Nl* without prior contact), p

< 0.001; Figure 1 C). In *Nv* females, heterospecific courtship had no effect on the subsequent duration of courtship by conspecific males (Mann–Whitney U test: U = 2283, n = 69 (*Nv* courted) and 63 (*Nv* without prior contact), p = 0.62; Figure 1D).

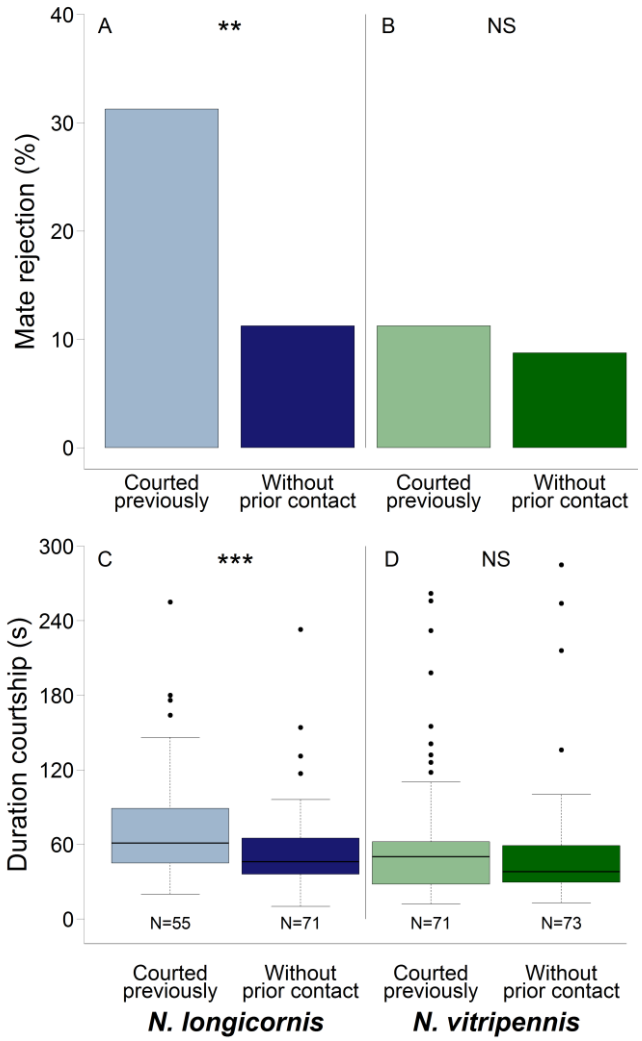

Figure 1 Females of *Nasonia longicornis* (**A**) rejected conspecific males more often and (**C**) consented to mating a conspecific male only after prolonged courtship when having been courted previously by a heterospecific male. In *N. vitripennis* females, heterospecific courtship had no effect on (**B**) female conspecific mate rejection, or (**D**) on the duration of courtship of the conspecific males. Boxplots display median (horizontal line within the box), lower and upper quartile (box margins), maximum/minimum range (whiskers; <1.5× above box height), and outliers (dots; ≥1.5× above box height). Asterisks indicate significant differences: Chi-square test (**A,B**) and Mann–Whitney U test (**C,D**): ** p < 0.01, *** p < 0.001, NS: Not significant.

Aggression

Aggression by heterospecific males had no effect on *Nl* female mate rejection (Chi-square test: $\chi_1 = 0.24$, $N = 32$ (aggressive contact) and 48 (non-aggressive contact), $p = 0.62$) or the duration of subsequent conspecific courtship (Mann–Whitney U test: U = 812, $N = 21$ (aggressive) and 32 (non-aggressive), $p = 0.23$).

DISCUSSION

Nl females were more reluctant to mate a conspecific after having been courted by a heterospecific male. This was reflected in both an increased conspecific mate rejection rate and an increased duration of courtship needed to induce receptivity in couples in which copulation took place. This reluctance to mate a conspecific was not a result of the aggressive attacks exhibited by *Nv* males towards *Nl* females. Hence, it is likely that *Nl* females use previous contact with and/or courtship by heterospecific males to assess the future risk of mismating. *Nl* females alerted by previous heterospecific courtship adjust their general mating behaviour and show an increased rejection of even conspecific males in subsequent courtship encounters. However, whether the mere contact with a heterospecific male, e.g., antennal contact, or the exposure to heterospecific courtship induces this adjustment remains to be investigated.

Rejecting conspecific males more frequently and consenting to conspecific copulation only after prolonged courtship likely results in *Nl* females losing valuable time that could otherwise be spent on the location of new host patches. In addition, it potentially increases the risk of leaving the natal host patch unmated. However, several males usually emerge from a host patch, and unmated females are potentially courted by several males prior to dispersal. In addition, at least in *Nv*, females become restless and switch to host-seeking behaviour only after mating (King et al., 2000; Ruther et al., 2014). Consistently, unmated *Nv* females have been rarely found to oviposit on new host patches in nature (Grillenberger et al., 2008).

In contrast to *Nl* females, conspecific mate acceptance in *Nv* females was not affected by prior heterospecific courtship. A possible reason for this difference between the two species is that *Nv* females follow a different strategy to counteract the costs arising from interspecific mating. *Nv* females that have copulated with a heterospecific (*N. giraulti, Ng*) male show an increased willingness to remate with a conspecific. By remating, *Nv* females are able to produce female offspring numbers that are similar to those found in females that mated only once with a conspecific (Ruther et al., 2014). However, whether females of *Nl* exhibit similar remating adjustments needs further investigation. Furthermore, the costs of heterospecific copulation imposed on females of the two species likely differ in another aspect. While cytoplasmic incompatibility induced by the infection with strains of *Wolbachia* leads to a conversion to male offspring in *Nv*, it predominantly leads to mortality in *Nl* (Tram et al., 2006). As a result, *Nl* females that have copulated with a heterospecific male most likely suffer higher fitness costs than *Nv* females that consented to interspecific copulation.

Mate discrimination is a plastic behavioural trait in *Nasonia*. Females adjust their mate acceptance rate according to their internal state and previous experience: they become less choosy with age (*Nv* and *Ng*) (Ruther et al., 2014; Mair et al., 2017), refrain from mating when they have already mated with a conspecific (*Nv*) (van den Assem & Visser, 1976), show increased remating frequencies when having mated with a heterospecific male before (*Nv*) (Ruther et al., 2014), and as shown here, are able to adjust mate acceptance in response to having experienced courtship by heterospecific males (*Nl*).

Plasticity in mate discrimination in response to being held in the presence of heterospecific mating partners has been demonstrated in various animal taxa. Females of the silverleaf whitefly *Bemisia tabaci* increase conspecific mate acceptance when males of a different biotype are present in the environment (Crowder et al., 2010). Females and males of three-spined sticklebacks (*Gasterosteus* spp.) become more discriminating against heterospecific partners when reared in mixed populations (Kozak & Boughman, 2009). Males of the Trinidadian guppies *Poecilia reticulata* and *P. picta* as well as males of the fruit fly *Drosophila persimilis* learn to distinguish between conspecific and heterospecific females

when held in mixed populations (Magurran & Ramnarine, 2003; Dukas, 2008). However, in contrast to *Nl*, individuals in these studies developed more accurate mate discrimination abilities in response to the presence of a reproductively interfering species. As females of *Nl* usually mate only once during their lifetime, a single mistake in mate choice is extremely costly for them, particularly if it is not, similar to in *Nv*, counteracted by increased remating after interspecific copulation. Instead of relying on the relatively slow process of learning by repeated experience, it may thus be more advantageous for *Nl* females to become more selective the moment that they recognize the presence of the interfering species. Recent studies indicate that the four *Nasonia* species differ in far more behavioural and ecological aspects than assumed so far, and that species interactions are far more complex than described to date (Leonard & Boake, 2006, 2008; Niehuis et al., 2013; Ruther et al., 2014; Giesbers et al., 2016; Mair et al., 2017; Mair & Ruther, 2018). In general, the potential role that the behavioural plasticity of mate discrimination can play in interactions and dynamics among co-occurring species has until now been widely neglected. The *Nasonia* model system offers fruitful opportunities for future investigations of plastic mate discrimination and species interactions on evolutionary, ecological, and behavioural levels, as well as on the level of underlying neurobiological and physiological processes.

CONCLUSIONS

After having been unsuccessfully courted by a heterospecific male, females of *Nl* are more reluctant to mate with a conspecific. In contrast, females of *Nv* do not change their mate acceptance behaviour following unsuccessful heterospecific courtship. This difference in the plasticity of mate acceptance between the two species is reasonable, because *Nl* females likely face higher fitness costs when consenting to interspecific copulation, and *Nv* females counteract the costs of mismating additionally through increased re-mating rates.

SUPPLEMENTARY MATERIALS

The following are available online at http://www.mdpi.com/2075-4450/9/3/112/s1, **Table S1** Raw data.

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6. Lack of reinforcement by increased mate discrimination in females of the parasitoid wasp *Nasonia longicornis* reared in artificial sympatry with *N. giraulti* males

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ABSTRACT

The principle of reinforcement states that prezygotic reproductive isolation mechanisms should evolve among co-occurring species pairs as a result of fitness costs imposed on hybridising individuals through postzygotic reproductive isolation. Female mate discrimination is a substantial force strengthening prezygotic reproductive isolation in various animal taxa. We investigated reinforcement through increased female mate discrimination by rearing parasitoid wasps of *Nasonia longicornis* (*Nl*) in artificial sympatry with males of the naturally allopatric species *N. giraulti* (*Ng*). We show evidence that the presence of *Ng* males in sympatry likely imposed fitness costs on *Nl* females through reproductive interference by interspecific courtship and copulation. In addition we show that in *Nl* females, remating with a conspecific male does not compensate completely for the fitness costs arising from previous interspecific copulation. Mate discrimination in *Nl* females, however, did not increase in response to prolonged rearing (53 generations) of *Nl* in the presence of interfering *Ng* males.

INTRODUCTION

When two closely related species that are reproductively isolated by postzygotic isolation mechanisms co-occur in sympatry, prezygotic isolation barriers should evolve to avoid fitness costs imposed on them by interspecific courtship and copulation. These costs include the time and energy spent on courtship displays and copulation (reproductive interference) and the costs of producing unfit hybrids (Orr & Presgraves, 2000; Gröning & Hochkirch, 2008). The strengthening of prezygotic reproductive isolation barriers as a consequence of fitness costs imposed on hybridising individuals in areas of species overlap is termed reinforcement (Butlin, 1987; Noor, 1999; Servedio & Noor, 2003; Servedio, 2004). Understanding reinforcement and the evolution of reproductive isolation mechanisms in sympatry are key to understanding the process of speciation (Servedio & Noor, 2003; Ortiz-Barrientos et al., 2009). Reinforcement can either occur during sympatric speciation or when two species which have been geographically isolated for a considerable amount of time and which lack complete prezygotic reproductive isolation come into contact in zones of overlap (Liou & Price, 1994; Bolnick & Fitzpatrick, 2007). One way to study reinforcement is to bring two allopatric populations or closely related species into contact in artificial sympatry. This can be achieved either in controlled experiments (Higgie et al., 2000; Urbanelli et al., 2014) or by studying natural populations after the accidental anthropogenic introduction of animals into new environments (Remnant et al., 2014).

Reproductive isolation between co-occurring species can be reinforced through various mechanisms (Servedio & Noor, 2003), for example by shifting the time or place where copulations happen or changing signals used in sexual communication (reproductive character displacement; (Howard, 1993; Higgie et al., 2000; Urbanelli et al., 2014). In addition, reinforcement can be achieved by increasing the ability to discriminate between mating partners (Liou & Price, 1994; Hudson & Price, 2014) or by counteracting mistakes in mate choice through post-copulatory mechanisms such as cryptic female choice (Eberhard, 1991; Matute, 2010).

The genus *Nasonia* is an excellent model system to study the evolution of reproductive isolation in parasitoid wasps. The ecology of *Nasonia* is well understood, the wasps most likely interact on shared host patches, and the species distribution allows for the establishment of artificial sympatry between naturally allopatric species. The genus consists of four species: *N. vitripennis* (*Nv*) is cosmopolitan and co-occurs with all other *Nasonia* species (Darling & Werren, 1990; Grillenberger, van de Zande, et al., 2009; Raychoudhury, Desjardins, et al.,

2010; Raychoudhury, Grillenberger, et al., 2010). *Nv* often develops in microsympatry, i.e. inside the same host individual, with them (Grillenberger, van de Zande, et al., 2009). *Nasonia* wasps are gregarious and several adults emerge from the same host which results in interspecific interactions and reproductive interference in areas of overlapping distribution. *N. giraulti* (*Ng*) is restricted to the eastern part of North America and *N. longicornis* (*Nl*) is restricted to the western part of North America (Darling & Werren, 1990). *Ng* and *Nl* do not occur in sympatry in nature. The forth species, *N. oneida* (*No*), is merely known from two locations in New York State (Raychoudhury, Desjardins, et al., 2010), and only little information on the ecology and behaviour of this species exists to date. All *Nasonia* species (except for the species pair *Ng*-*No*) are reproductively isolated by post-zygotic cytoplasmic incompatibility resulting from the infection with different strains of the intracellular bacterium *Wolbachia* (Bordenstein et al., 2001). Males of all *Nasonia* species show an elaborate courtship display: When a male encounters a female, he mounts the female, positions himself on the female's head and thorax and starts moving his mouthparts over the female's antennae, a behaviour called head-nodding. This behaviour is accompanied by stroking movements with the male's legs over the female's head and eyes (van den Assem & Vernel, 1979; van den Assem, Gijswijt, et al., 1980; van den Assem, Jachmann, et al., 1980; van den Assem et al., 1981; van den Assem & Werren, 1994). During head-nodding, an oral male aphrodisiac is transferred to the female's antennae which triggers the female's receptivity signal and copulation follows (van den Assem, Jachmann, et al., 1980; Ruther et al., 2010). Males readily engage in courtship and copulation with females of all other *Nasonia* species (Giesbers et al., 2013; Buellesbach et al., 2014, 2018; Mair et al., 2017). In contrast, females discriminate between conspecific and heterospecific mating partners, but the level of discrimination differs among species (Giesbers et al., 2013; Buellesbach et al., 2014). The information used by females to discriminate between mating partners is unclear, but is likely encoded in the males' courtship display which differs among the *Nasonia* species (van den Assem & Vernel, 1979; van den Assem, Gijswijt, et al., 1980; van den Assem & Werren, 1994) or the composition of the male aphrodisiac. Among the *Nasonia* species, *Nv* females discriminate most against heterospecific mates (Giesbers et al., 2013; Buellesbach et al., 2014; Ruther et al., 2014; Mair et al., 2017, 2018). *Nv* females usually mate only once during their life (Holmes, 1974; van den Assem & Visser, 1976; Grillenberger et al., 2008). In cases *Nv* females copulate with a heterospecific male by mistake, however, they remate with a conspecific to counteract the costs of mismating (Ruther et al., 2014). Remated females are able to produce offspring clutch sizes and sex ratios which are similar to those of females having mated only once with a

conspecific (Ruther et al., 2014). It is likely that the strong mate discrimination and increased remating after interspecific copulation have evolved in *Nv* females as a result of microsympatry with the other *Nasonia* species.

The different *Nasonia* species differ in several aspects associated with the species' reproductive behaviour. While males of *Nv* are territorial and copulation in *Nv* happens after emergence from the host, males of *Ng* are not territorial and copulation in *N*g happens mostly inside the host prior to emergence (van den Assem, Gijswijt, et al., 1980; van den Assem, 1996; Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013; Ruther et al., 2014; Mair & Ruther, 2018). In *Ng*, within-host-mating rates of up to 100 percent have been reported (Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013; Ruther et al., 2014). Males of *Nl*, on the other hand, show intermediate levels of within-host-mating (on average 5 to 24 percent) and, similar to *Nv* males, exhibit aggression at the natal host after emergence (Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013). Whether they establish a territorial system similar to that of *Nv* remains unclear so far. Among all *Nasonia* species, *Nl* females are least discriminating against heterospecific mating partners (Giesbers et al., 2013; Buellesbach et al., 2014). Because *Ng* and *Nl* are not sympatric in nature, the two species offer an ideal opportunity to study reinforcement of reproductive isolation by artificial sympatry.

We investigated reinforcement by increased mate discrimination in females of *Nl* when reared in microsympatry with males of *Ng*. For this purpose we first assessed the potential fitness costs imposed on *Nl* females by the presence of *Ng* males. We investigated the likelihood of interspecific mating between *Nl* females and *Ng* males and the occurrence and effectiveness of remating as a countermeasure for mismating in *Nl* females. We then set up a breeding scheme in which *Nl* was reared in artificial microsympatry with *Ng* males. After several generations of artificial microsympatry, we evaluated whether mate discrimination in *Nl* females had increased.

MATERIALS AND METHODS

Rearing and wasp preparation

Experiments were conducted with the two strains NLMN8510* (*Nl*) and NgMIX (*Ng*; (van de Zande et al., 2014). All wasps were reared on pupae of the bottle fly *Lucilia caesar* (L.) at 25 °C under a 16:8 L:D light regime. For the preparation of experimental wasps, fly

puparia were opened two to three days prior to the expected emergence of the wasps and wasp pupae were isolated and kept singly in 1.5 ml microcentrifuge tubes until being used in the experiments. Every morning, newly eclosed wasps were sexed. At the day of eclosion, wasps were defined as being zero days old. By isolating wasps prior to eclosion it was ensured that the wasps used in experiments were virgin and inexperienced, i.e. they had no previous direct contact to other adult wasps. The assignment of single wasps to experiments and treatments was randomised.

Potential costs of sympatry

Likeliness of interspecific copulations

The likelihood of interspecific copulations between *Nl* females and males of the naturally allopatric species *Ng* was investigated by evaluating *Ng* male mate discrimination and female readiness to mate with *Ng* males, respectively. Because mate discrimination in *Nasonia* females is usually dependent on female age (Ruther et al., 2014; Mair et al., 2017), female mate discrimination was assessed for zero-day-old and two-day-old females separately. In line with previous studies on mate discrimination (Giesbers et al., 2013; Buellesbach et al., 2014; Ruther et al., 2014; Mair et al., 2017), we expected mate discrimination in *Nl* females to be relatively weak, and virtually absent in *Ng* males. Similar to *Nv* and *Ng* females (Ruther et al., 2014; Mair et al., 2017), we expected *Nl* female mate discrimination to decrease with age.

Courting males of *Nasonia* species respond to chemical cues present on the female's cuticle and thus exhibit courtship also towards dead females (Steiner et al., 2006; Buellesbach et al., 2013). In behavioural bioassays, two-day-old males of *Ng* were subjected to dead females of *Nl* and *Ng* and their readiness to mount, court and copulate with these dead females was recorded. In two separate experiments, *Ng* males were either subjected to one single female at a time (single choice) or to two females (one of each species) simultaneously (dual choice). One-day-old females were killed by freezing at -20°C, glued with non-toxic glue to filter paper in an upright position and allowed to thaw for 5 minutes before being used in the experiments. In the dual choice experiment, the two females were glued next to each other, facing in the same direction. The position of the females (either left or right) was alternated in each trial. Bioassays were conducted by putting a standard mating arena (diameter: 10 mm, height: 3 mm; (Ruther et al., 2000) over the dead female(s), releasing the experimental male into the arena, covering the arena with a cover slip and observing the male's behaviour for five minutes. Male behaviours were recorded in real-time via the software The Observer XT 9.0

(Noldus, Wageningen, The Netherlands). The time each male spent mounted on the dead female(s) was recorded and the number of trials with and without courtship (males exhibiting head-nodding behaviour or not) and the number of trials with and without copulation attempts were counted. In the dual choice experiment, measurements were taken for each dead female separately. 20 replicates were conducted each (*Nl* single choice, *Ng* single choice, dual choice). The time that males spent mounted on females was compared between *Nl* and *Ng* females by means of a Mann-Whitney U test for the single choice experiment and a Wilcoxon signed-ranks test for paired samples for the dual choice experiment. The number of trials with and without courtship and copulation, respectively, were compared between the two species with 2x2 Chi-square tests.

Nl female mate discrimination was investigated by conducting mating trials with males of *Nl* and of *Ng*, respectively, and assessing the females' mate rejection rates. Mating trails were conducted by placing one male and subsequently one female into a standard mating arena and observing the couple for five minutes. A male was classified as rejected, when the female did not show receptivity during the observational time. Trials in which the male did not start courtship were discarded. Trials were conducted with zero-day-old and two-day-old females separately, resulting in a total of four treatments (two age classes, two species). Males were two days old. The number of trials in which the female consented to copulation/refused to copulate were compared between males of the two species with a 2x2 Chi-square test for zero-day-old and two-day-old females separately. The heterospecific mate rejection rates assessed in this experiment were also used as a baseline in the evaluation of a change in mate discrimination in the artificial sympatry experiment.

Fitness costs imposed by interspecific mating

The fitness costs imposed by heterospecific mating were further evaluated by assessing remating rates, clutch sizes and offspring sex ratios of differently mated *Nl* females. Females having mated with a heterospecific male are not able to produce hybrid offspring due to *Wolbachia*-induced cytoplasmic incompatibility (Bordenstein et al., 2001). In addition, in *Nl*, eggs fertilised by heterospecific sperm usually die and less than half of the eggs develop into adult male offspring (Bordenstein et al., 2003; Tram et al., 2006). *Nl* females thus face substantial fitness costs when choosing the wrong mating partner. In addition, remating rates and the consequences for offspring production have not been investigated in *Nl* so far. Because *Nl* and *Ng* do not occur in sympatry in nature, we expected remating rates to be similar in both, *Nl* females having previously mated with a conspecific and those having previously mated

with an *Ng* male. In addition, we expected remating in *Nl* females to be less effective than in *Nv* females in reducing costs imposed by mismating. More specifically, due to the death of eggs fertilised by heterospecific sperm, we expected offspring clutch sizes to be smaller in remated females than in females having mated only with a conspecific. Due to local mate competition among males at the natal host patch after emergence, *Nasonia* females usually produce strongly female-biased offspring sex ratios (15 to 20 percent males) when ovipositing alone (Hamilton, 1967; Werren, 1983; Shuker et al., 2006; Burton-Chellew et al., 2008). Because eggs in remated females are expected to be partly fertilised by heterospecific sperm, we expected remated females to produce higher sex ratios (more male offspring) than females having mated only with a conspecific.

To assess remating rates, zero-day-old and two-day-old females of *Nl* were allowed to mate with either a conspecific or a heterospecific male in a standard mating arena. Females that refrained from mating for five minutes were discarded. After successful copulation, the respective male was allowed to unmount the female before being removed carefully from the arena and the female was given five minutes to remate with a conspecific male. For each female it was noted whether she remated with the conspecific male or not. The number of females having consented to remating was compared between females having previously mated with a conspecific and those having previously mated with a heterospecific male with a 2 x 2 Chi-squared test for zero-day-old and two-day-old females separately.

Fitness costs imposed by interspecific mating were investigated by mating *Nl* females either only once with a conspecific male, or mating *Nl* females with one heterospecific male followed by subsequent remating with a conspecific and determining the females' offspring clutch size and sex ratio. Matings were conducted in standard mating arenas and each couple was given five minutes to copulate. After having mated with a heterospecific male, the respective female was given another five minutes to remate with a conspecific. If copulation did not happen during observational time, the couple was discarded. All females used in the experiment were zero to two days old and males were zero to three days old. Each male was used only once for mating and assignment of individuals to treatments was randomised. Mated females were subsequently put into one petri dish each, supplied with ten fresh hosts for oviposition and kept at rearing conditions for 48 ± 3 hours. Offspring was counted and sexed when all offspring had emerged from the host (approximately three weeks after oviposition). In addition, host puparia were opened and wasp pupae that had not fully developed and eclosed were counted, sexed and included in the clutch size and calculation of offspring sex ratio as

well. Clutch sizes and offspring sex ratios were compared between treatments with Mann-Whitney *U* tests.

Artificial sympatry

Two experimental breeding lines were set up to investigate the effect of microsympatry with *Ng* males on *Nl* female mate discrimination. In the artificial sympatry line, wasps of *Nl* were reared on fly pupae having been already pre-parasitised by virgin females of *Ng* producing only male offspring. By using virgin *Ng* females we ensured that *Nl* wasps were not replaced by *Ng* in the artificial sympatry line and that only *Nl* females were tested in mate discrimination trials. By using virgin *Ng* females we furthermore intended to increase the selection pressure on *Nl* females by increasing the number of courting *Ng* males present in the host before emergence and in the environment after emergence. *Ng* males were given a developmental head start of 24 hours to ensure that *Ng* males had already eclosed prior to females of *Nl* and to give *Ng* males an advantage in the competition with males of *Nl*. For control, a second breeding line was set up following the same rearing procedures but without pre-parasitisation of the hosts.

Wasps were reared under standard rearing conditions in four petri dishes per generation containing 40 fly pupae each. In each generation, at least 15 *Nl* females were transferred from each petri dish to fresh hosts by exchanging the lids of the petri dishes on which wasps typically like to sit. To keep the genetic diversity of the lines as high as possible, wasps were transferred in such a way that females of each old petri dish were distributed to all four new petri dishes, mixing up females in each generation. Hosts were prepared one day before the transfer of the wasps by thawing frozen fly puparia for 30 minutes at 30°C. Those freeze-killed hosts are well accepted by *Nasonia* wasps for oviposition. After thawing, hosts were counted and distributed to petri dishes. In the artificial sympatry line, 11 virgin *Ng* females were subsequently put into each new petri dish and allowed to oviposit under rearing conditions for 24 ± 3 hours. Hosts of the control line were likewise kept under rearing conditions during that time but without adding *Ng* females. The position of the petri dishes in the climate chamber was randomised. After pre-parasitisation, *Ng* females were removed from the hosts and *Nl* wasps from both breeding lines were transferred to the respective new petri dishes. These procedures were repeated in each generation.

Changes in *Nl* female mate discrimination in the two breeding lines over time were assessed in generations 23, 30, 36, 39 and 54 following experimental procedures as described above for mate discrimination bioassays. The mate discrimination rates assessed prior to

setting up breeding lines were used as a baseline reference point of the initial population to which mate discrimination rates of the following generations could be compared. Similar to the procedure described above, zero-day-old and two-day-old females were tested separately, because mate discrimination has been shown to decrease in *Nasonia* wasps with increasing age (Ruther et al., 2014; Mair et al., 2017). As a control for changes in general mate acceptance, conspecific mate rejection rates were assessed for each age class and generation in both the artificial sympatry line and the control line.

We expected *Nl* females to suffer from considerable fitness costs due to reproductive interference with *Ng* males in artificial sympatry. More specifically, we expected *Ng* males to court and try to copulate with *Nl* females both inside the host prior to emergence and outside the host after emergence. Consequently, we expected to find an increase in mate discrimination in females of the artificial sympatry line, but not in the control line.

Heterospecific mate rejection data was analysed by fitting a logistic regression model on the binary response 'mated/rejected' using the R function glm() with binomial distribution and logit link function. Age (zero-days-old, two-days-old), line (artificial sympatry, control) and generation (23, 30, 36, 39 and 54) as well as the interaction terms 'line * generation' and 'age * line * generation' were included as predictors. Because the mate discrimination rates prior to the setup cannot be attributed to either of the breeding lines, they were excluded from the model. Model residuals were checked with the R package DHARMa (Hartig, 2018). All analyses were done in R version 3.5.0 (R Development Core Team, 2018).

RESULTS

Potential costs of sympatry

Likeliness of interspecific copulations

Males of *Ng* did not discriminate between dead conspecific females and females of *Nl*. In both, single choice and dual choice experiments, they spent a similar amount of time mounted on dead conspecific as on dead heterospecific females (N = 20 each; single choice: Mann-Whitney U test, $U = 209$, $P = 0.82$; dual choice: Wilcoxon signed-ranks test, $T = 93$, $P = 0.95$; Fig. 1 a, b).

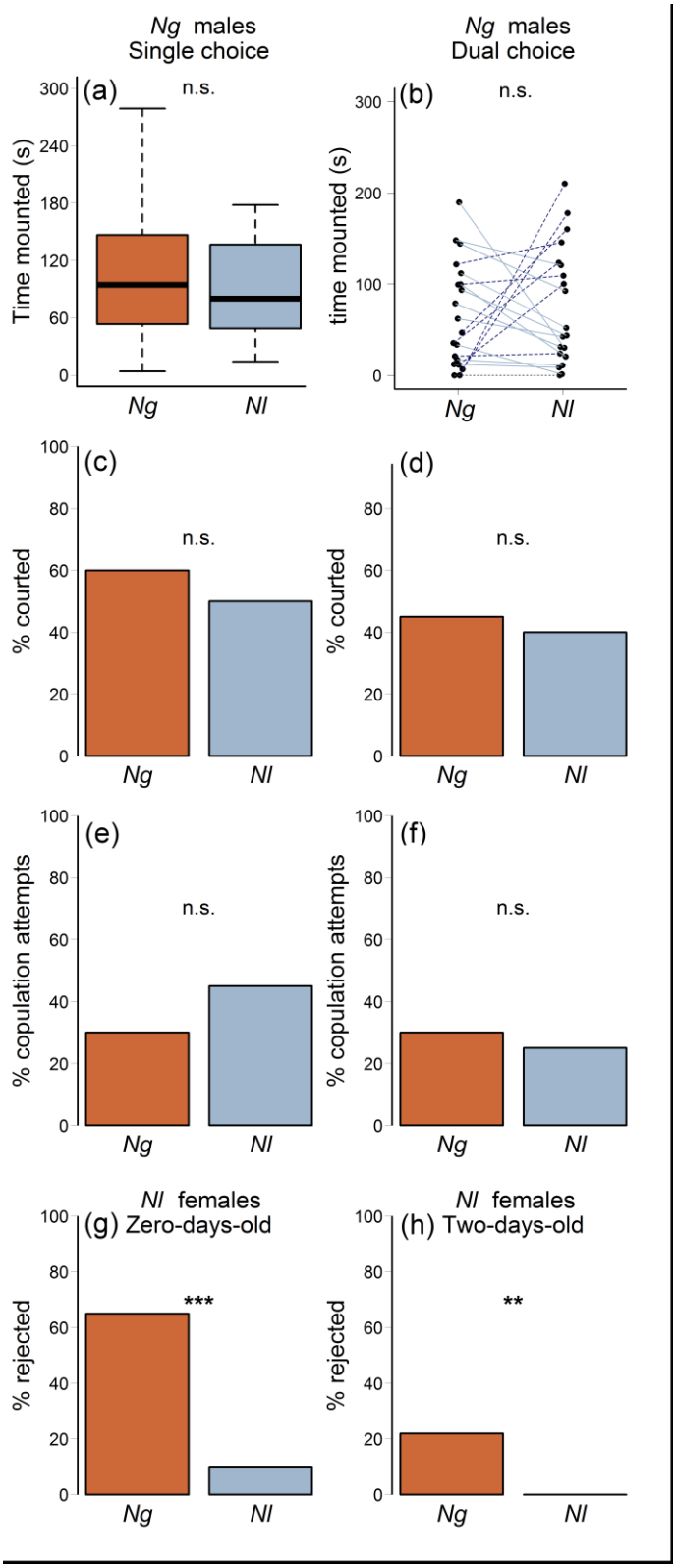

Figure 1 In behavioural bioassays, *Ng* males did not discriminate between dead conspecific females and dead *Nl* females: (a, b) time mounted, (c, d) proportion courtship and (e, f) frequency copulation attempts. Results are shown for (a, c, e) single choice and (b, d, f) dual choice experiments. (g, h) Although *Nl* females discriminated between conspecific males and *Ng* males, they consented to mating with heterospecific males in 35 (zero-day-old females) and 78 (two-day-old females) percent of samples. Boxplots display median (horizontal line within the box), lower and upper quartile (box margins), maximum/minimum range (whiskers) and outliers (dots; 1.5

above box height). Asterisks indicate significant differences. (a) Mann-Whitney *U* test, (b) Wilcoxon signed-ranks test and (c – h) 2 x 2 Chi-square tests; **: $P < 0.01$, ***: $P < 0.001$, n.s.: not significant.

In addition, *Ng* males showed courtship and copulation attempts in both experiments equally often with con- as with heterospecific dead females (2 x 2 Chi-squared tests, $N = 20$ each; single choice: courtship: $\chi^2_1 = 0.40$, $P = 0.53$, copulation attempts: $\chi^2_1 = 0.96$, $P = 0.33$; dual choice: courtship: $\chi^2_1 = 0.10$, $P = 0.75$; copulation attempts: $\chi^2_1 = 0.13$, $P = 0.72$; Fig. 1 c - f).

Females of *Nl* discriminated between con- and heterospecific mating partners (2 x 2 Chi-squared tests, $N = 40$ each; zero-day old females: $\chi^2_1 = 25.8$, $P < 0.0001$; two-day-old females: $\chi^2_1 = 9.65$, $P = 0.002$; Fig. 1 g, h). Nevertheless, 35 percent of the zero-day-old females and 80 percent of the two-day-old females showed receptivity and copulated with heterospecific mating partners.

Fitness costs imposed by interspecific mating

Remating in both zero-day-old and two-day-old females of *Nl* happened equally often after having mated with a conspecific male as compared to having mated with a male of *Ng* (2 x 2 Chi-squared tests, zero-day-old females: $N_{\text{conspecific}} = 36$, $N_{Ng\ \text{male}} = 14$, $\chi^2_1 = 1.55$, $P = 0.21$; two-day-old females: $N_{\text{conspecific}} = 39$, $N_{Ng\ \text{male}} = 32$, $\chi^2_1 = 0.06$, $P = 0.80$; Fig. 2 a, b). Remating rates ranged from 77.8 (zero-day old conspecific) to 92.9 percent (zero-day-old *Ng* male).

Females of *Nl* having mated with a *Ng* male prior to mating a conspecific produced higher offspring sex ratios, i.e. a higher relative number of male offspring, than females having only mated with a conspecific ($\text{mean}_{Ng+Nl} = 0.41$, $\text{mean}_{\text{only } Nl} = 0.14$; Mann-Whitney *U* test, $N_{Ng+Nl} = 33$, $N_{\text{only } Nl} = 35$, $U = 862$, $P < 0.001$; Fig. 2 c). Clutch sizes produced by females having mated with a *Ng* male prior to mating a conspecific did not differ from clutch sizes produced by females having only mated with a conspecific (Mann-Whitney *U* test, $N_{Ng+Nl} = 33$, $N_{\text{only } Nl} = 35$, $U = 703.5$, $P = 0.12$; Fig. 2 d).

Artificial sympatry

In both breeding lines, heterospecific mate rejection rates increased over time (logistic regression model: effect of generation, $\chi^2_3 = 24.53$, $P < 0.0001$; Table 1, Fig. 3). Like in the initial population, zero-day-old females rejected heterospecific males more often than two-day-old females (logistic regression model: effect of female age, $\chi^2_1 = 79.42$, $P < 0.0001$). This age effect was present in both lines in all generations except for generation 39 in the artificial

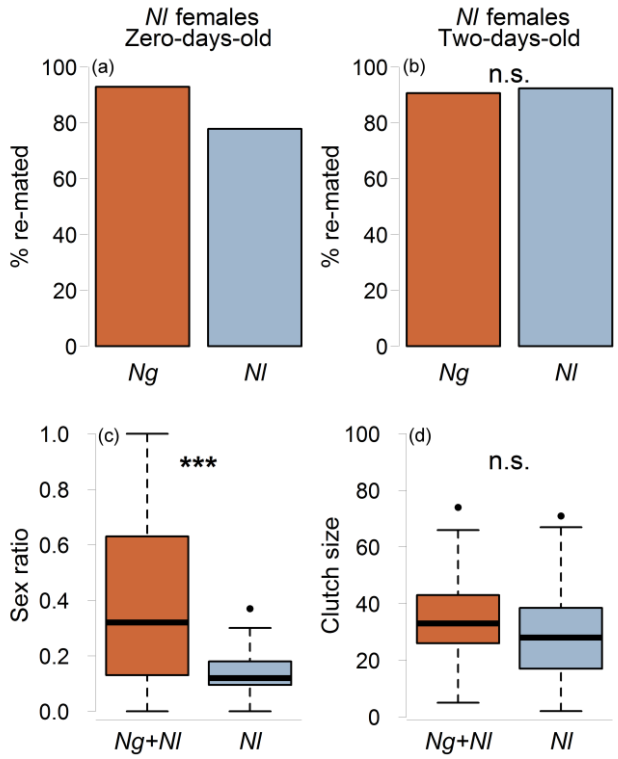

Figure 2 (a, b) Remating rates in zero-day-old and two-day-old females did not differ between females having first mated with a conspecific male compared to having first mated with an *Ng* male. (c) Females having mated with an *Ng* male before mating a conspecific produced a higher proportion of male offspring than females having mated only with a conspecific. (e) Heterospecific mating did not affect the total number of offspring produced. Boxplots display median (horizontal line within the box), lower and upper quartile (box margins), maximum/minimum range (whiskers) and outliers (dots; 1.5 above box height). Asterisks indicate significant differences (2 x 2 Chi-square tests (a, b) and Mann-Whitney *U* test (c, d): ***: $P < 0.001$, n.s.: not significant.

Table 1 ANOVA table of the logistic regression model on heterospecific mate rejection in the two breeding lines of the artificial sympatry experiment. female age: female age class (zero-day-old versus two-day-old females), line: breeding line (artificial sympatry versus control), generation (23, 30, 36, 39 and 54).

	Df	Deviance	Residual Df	Residual Deviance	*P*
NULL			462	620.52	
female age	1	79.43	461	541.10	< 2.2e-16 ***
line	1	1.52	460	539.57	0.218
generation	3	24.53	457	515.04	1.935e-05 ***
line:generation	3	5.62	454	509.43	0.132
female age:line:generation	7	25.27	447	484.16	0.00068 ***

sympatry line in which two-day-old females exhibited increased heterospecific mate rejection comparable to that of zero-day-old females. The change in the age effect over time thus differed significantly between the two breeding lines (logistic regression model: effect of interaction term female age * line * generation, $\chi^2_7 = 25.27$, $P < 0.001$). During the experiment, conspecific mate rejections rates never exceeded 20 percent.

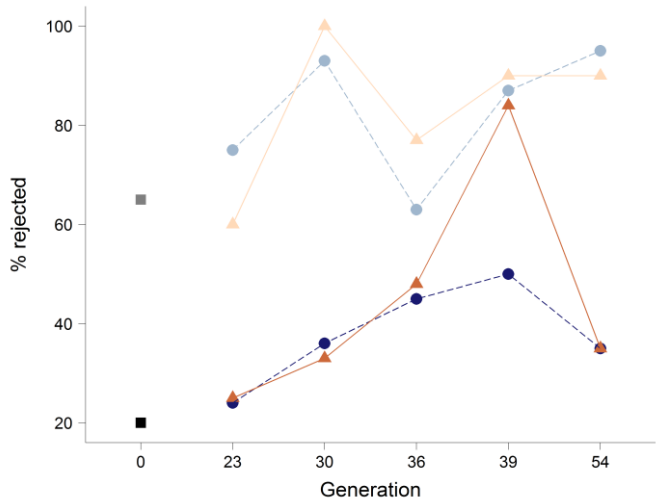

Figure 3 Heterospecific mate rejection rates exhibited by females in the course of the artificial sympatry experiment. Mate rejection rates for generation zero (initial population) are displayed for visual reference but were not included in the statistical analysis (see Table 1). Squares: initial population (grey: zero-day-old females, black: two-day-old females), circles with dashed line: control line (dark blue: two-day-old females, light blue: zero-day-old females), triangles with solid line: artificial sympatry line (dark orange: two-day-old females, light orange: zero-day-old females).

DISCUSSION

The results of our controlled mating trials indicated that *Nl* females in the artificial sympatry line likely faced the risk of fitness costs imposed on them by courtship and copulation with *Ng* males. *Ng* males readily courted and copulated with *Nl* females, and *Nl* female mate discrimination was not absolutely accurate. 35 percent of zero-day-old and 78 percent of two-day-old *Nl* females consented to copulation with *Ng* males. Remating rates in *Nl* females in our experiments were relatively high irrespective of whether the first male was conspecific or heterospecific. Nevertheless, remating in *Nl* females did not entirely counteract the costs of previous mismating. Remated females produced higher numbers of male offspring (on average more than 40 percent males) as compared to females having mated only once with a conspecific (on average 14 percent males).

Despite these findings, mate discrimination in *Nl* females reared in artificial sympatry with *Ng* males did not increase significantly as compared to mate discrimination in *Nl* females of the control line. Instead, heterospecific mate rejection increased over time in both breeding lines. We suppose that the general increase in heterospecific mate rejection during the experiment resulted from factors not measured in our experimental procedures. In addition, except for the high heterospecific mate rejection rate of two-day-old females in the artificial sympatry line in generation 39, mate rejection rates in both lines were surprisingly similar and followed the same fluctuation pattern. The cause of the high heterospecific mate rejection of two-day-old females in generation 39 remains unclear and we conclude that the changes in *Nl* female mate discrimination we observed did not result from artificial sympatry with *Ng*.

There are several possible reasons, why artificial sympatry with *Ng* males did not result in pronounced female discrimination against them. First of all, mate discrimination in *Nl* females, particularly in young females, has turned out to be stronger than thought so far. Earlier studies reporting low heterospecific mate rejection in *Nl* females were conducted with two- to three-day-old females (Giesbers et al., 2013; Buellesbach et al., 2014). Younger (zero-day-old) *Nl* females have been shown only lately to exhibit more than 90 percent heterospecific mate rejection when confronted with *Nv* males (Mair et al., 2018). This age effect in mate discrimination showed also in our experiments and is consistent with other *Nasonia* species (Ruther et al., 2014; Mair et al., 2017). Nevertheless, we found 35 percent of zero-day-old *Nl* females and 55 percent of two-day-old females consenting to copulation with an *Ng* male, still leaving space for stronger mate discrimination to evolve.

Second, *Nl* females could have counteracted mistakes in mate choice by increasing remating rates after interspecific copulation instead of increasing heterospecific mate rejection. Although remating rates were generally high in *Nl* females before setting up the breeding lines, females did not remate more often when having copulated with a heterospecific male as compared to when having copulated with a conspecific male before. Remating is increased in *Nv* females after heterospecific copulations and this counteracts the costs of mismating successfully (Ruther et al., 2014). However, in contrast to *Nv* females, our results show that in *Nl* females consenting to interspecific copulation leads to higher number of male offspring and thus fitness costs despite remating with a conspecific. An increase of the already high remating rates would have therefore been relatively ineffective in mitigating costs of mismating.

Third, it is possible that instead of increasing mate discrimination, *Nl* females evolved mechanisms to avoid fertilisation by heterospecific sperm after interspecific copulation.

Females of *Nv* usually show first male sperm precedence during the fertilisation of eggs (Holmes, 1974). Nevertheless, when the first mating partner has been heterospecific, *Nv* females are able to use the second (conspecific) male's sperm to fertilise their eggs and produce female-biased offspring sex ratios similar to those produced by females having mated only with a conspecific (Ruther et al., 2014). How remated *Nv* females are able to achieve the successful fertilisation of eggs by conspecific sperm is unknown, however. Possible mechanisms include, among others, the female's differential choice of sperm during fertilisation and the advantage of conspecific sperm in sperm competition due to specific sperm characteristics, e.g. the length of the flagellum, or additive components of the ejaculate provided by the copulating males (Eberhard, 1991; Birkhead, 1998).

Fourth, there still remains the possibility that the selection pressure imposed by *Ng* males on *Nl* females was not as high as we had expected based on the results of our behavioural bioassays. All behavioural bioassays were conducted under exclusion of male-male competition or any interspecific interactions that possibly occur at a shared host patch. In *Nv*, males exhibit an elaborate territorial system at the host from which they emerge (van den Assem, Gijswijt, et al., 1980; Mair & Ruther, 2018). The territorial male defends his territory aggressively against all challenging subordinate males. *Nl* males show site fidelity on the host after emergence and are involved in a similar number of aggressive male-male interactions on the natal host as *Nv* males (Leonard & Boake, 2006). Although this needs further investigation, it is thus possible that *Nl* males exhibit territoriality similar to *Nv* males. As a consequence, in microsympatry with *Ng*, they could have chased *Ng* males off their territory, thus preventing or reducing *Ng* male-*Nl* female interactions. *Ng* males are not territorial and disperse right after emergence from the host (Leonard & Boake, 2006; Mair & Ruther, 2018). They are therefore most likely easily expelled from male territories.

Fifth, it is possible that *Nl* males increased their mating success by switching to sneaking behaviour in a crowded environment. Breeding lines were kept in petri dishes, a relatively confined space which lacks complex environmental structures. In *Nv*, sneaking is a common strategy among subordinate males (Giesbers et al., 2013; Mair & Ruther, 2018). *Nasonia* males are smaller than the females and, at least in *Nv*, it happens frequently that two or more males mount one female simultaneously. In such a situation, one male usually positions himself at the females head and performs courtship while the others struggle for a position on the female's abdomen. When the female finally shows receptivity by opening the genital orifice, one of the non-courting males sneaks in and copulates. In behavioural bioassays with *Nv*, sneaking subordinate males succeeded in copulation similarly often as territorial males

(Mair & Ruther, 2018). In contrast, in bioassays investigating the mating behaviour of *Nv* and *Ng* males competing for a female, *Ng* males never exhibited sneaking behaviour (Giesbers et al., 2013). Sneaking by *Nl* males therefore has the potential to represent an additional strategy to mitigate costs imposed by heterospecific males in microsympatry.

Finally, *Ng* males exhibit within-host-mating rates of up to 100 percent (Ruther et al., 2014) and when setting up the experiment, we expected that *Ng* males would try to court and copulate with *Nl* females inside the host prior to emergence. A recent study with the species pair *Nv*-*Ng* showed, however, that within-host-mating in *Ng* is reduced substantially in multiparasitised hosts (Giesbers et al., 2016). In these hosts, *Nv* males bite exit holes into the fly puparium from which *Ng* females are able to escape before having mated with a conspecific male inside. If *Nl* males act similarly, interspecific courtship and copulation by *Ng* males inside the host could have been reduced remarkably.

In conclusion, reproductive isolation between *Nl* and *Ng* was not reinforced in our experiment through increased heterospecific mate rejection in *Nl* females having been subjected to prolonged breeding in microsympatry with *Ng* males. Whether the females adapted to the selection pressure imposed on them through other mechanisms or whether the selection pressure itself was not strong enough to induce reinforcement remains unclear. It is likely, however, that the complex repertoire of behaviours exhibited by the wasps at the natal host patch affects substantially the interspecific interactions and selection pressures in microsympatry.

General discussion

The behavioural repertoire of *Nasonia* is far more diverse and the species interactions are far more complex than previously thought. In virtually all stages of their adult life, the *Nasonia* species differ in the behavioural strategies they employ to reach their goals. Several of these differences likely evolved to counteract fitness costs arising from interspecific copulations. *Nasonia* species are reproductively isolated by postzygotic cytoplasmic incompatibility resulting from the infection with different strains of the intracellular bacterium *Wolbachia* (Breeuwer & Werren, 1990; Bordenstein et al., 2001). Females having mated with a heterospecific male are not able to produce hybrid female offspring and instead produce all-male broods, similar to virgin females (Breeuwer & Werren, 1990; Tram et al., 2006). It has been argued earlier (Bordenstein et al., 2001) that postzygotic reproductive isolation caused by *Wolbachia* infections occurred early in the stage of speciation in *Nasonia*. It is thus likely that reinforcement has been a driving force in shaping the differences in reproductive behaviour among the *Nasonia* species observed at present.

Males of the different *Nasonia* species differ substantially in the strategies they employ to gain mating opportunities. Males of *Nv* emerge prior to females, establish a relatively stable territorial system at the natal host patch, and court and copulate with females emerging from the hosts later (van den Assem, Gijswijt, et al., 1980; van den Assem, 1986; Mair & Ruther, 2018 (chapter3)). In contrast, *Ng* wasps mate inside the host prior to emergence, females emerge prior to males, and males are not territorial (Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013; Ruther et al., 2014; Mair & Ruther, 2018 (chapter 3)). Both, *Nv* and *Ng* males engage to the same degree in pheromone marking activities after emergence from the host (Mair & Ruther, 2018 (chapter 3)). The male abdominal sex pheromone is highly attractive for virgin females (van den Assem, Jachmann, et al., 1980; Ruther et al., 2007, 2008, 2010, 2014; Steiner & Ruther, 2009a; b; Niehuis et al., 2013). Applying territorial markings is thus obviously reasonable for *Nv* males. In *Ng*, male pheromone markings, although useless when all females emerge from the host mated, are likely adaptive when *Ng* develop in microsympatry (inside the same host) with *Nv*. Giesbers et al. (2016) have shown that in hosts multiparasitised by both *Ng* and *Nv*, a larger proportion of *Ng* females emerges as virgins. These virgin females are likely attracted by *Ng* male pheromone markings. Male behaviour at the natal host patch in the other two *Nasonia*

species, *Nl* and *No*, has not been studied in detail so far and only few characteristics have been described. Both species show intermediate within-host-mating rates (ca. 10% in *Nl* and 30 % in *No*; Drapeau & Werren, 1999; Leonard & Boake, 2006; Giesbers et al., 2013). *Nl* males exhibit aggression on the natal host after emergence and show site fidelity similar to *Nv* males (Leonard & Boake, 2006). Whether they also establish true territoriality needs further investigation, however. In addition, male marking behaviour in *Nl* and *No* has not been investigated to date. It has been shown recently, however, that *No* males produce on average ten times less sex pheromone than *Ng* males, and several *No* males lack the male sex pheromone completely (Diao et al., 2016). These results indicate that *No* males do not use the abdominal sex pheromone to attract females.

In chapter 4 (Mair et al., 2017), I have shown that, in contrast to *Nv* males, *Ng* males discriminate between conspecific and heterospecific females. In addition, I have shown that males of the two species rely on different chemical messengers to recognise conspecific females. While *Nv* males recognise females based solely on the females' cuticular hydrocarbons, *Ng* males require the complete cuticular lipid blend, including more polar lipids, to recognise conspecific females. In addition, my results indicate that *Ng* males use additional cues apart from the chemical messengers during mate recognition, e.g., visual, acoustic or tactile cues. Whether *Ng* male mate discrimination is strong enough to significantly affect reproductive isolation between *Nv* and *Ng*, and how mate discrimination, within-host-mating and the lack of territoriality in *Ng* together influence interspecific interactions and dynamics at shared host patches in nature remains to be investigated.

Another characteristic that differs between *Ng* and *Nv* and has been argued to have evolved as a countermeasure for reproductive interference is the additional sex pheromone component (4*R*,5*R*)-5-hydroxy-4-decanolide (RR) in *Nv* (Niehuis et al., 2013; Ruther et al., 2014). RR is virtually absent in all other *Nasonia* species, including *Ng* (Niehuis et al. 2013). Based on the presence of RR in the pheromone, *Nv* females are able to discriminate between conspecific and heterospecific male markings (Niehuis et al., 2013). This ability to discriminate is most likely adaptive in situations where both *Nv* and *Ng* (or presumably also *Nl*) mark the substrate to attract females on shared host patches.

In conclusion, the species differences that most likely act as prezygotic reproductive isolation mechanisms between *Nv* and *Ng* are understood quite well. The effects that these mechanisms have on reproductive interference under more natural circumstances, however, necessitate future research. Clearly, their role as effective reproductive isolation barriers needs validation.

In addition, more detailed studies on species differences and interactions among the other *Nasonia* species apart from female mate discrimination have come up only recently and may give valuable insights into the mechanisms allowing the species to co-occur in microsympatry in nature. Leonard & Boake (2006), for example, have found a negative correlation between within-host-mating rates and male aggression in a comparative approach in the species *Nv*, *Ng* and *Nl*. In addition, I have shown that females of *Nl* adjust their mate acceptance in response to previous courtship by *Nv* males (Mair et al., 2018 (chapter 5)). *Nl* females are able to adjust their reproductive behaviour in a flexible way to the circumstances they perceive at a given time and place. Females of *Nv*, in contrast, do not show this behavioural plasticity. Furthermore, in a first approach to study the dynamics happening in or at shared hosts, Giesbers et al. (2016) have shown that multiparasitisation with *Nv* decreases within-host-mating rates in *Ng* substantially. Based on their results, Giesbers et al. (2016) conclude that within-host-mating in *Ng* is a trait driven by the males' refusal to bite an exit hole into the fly puparium. When *Ng* develop in microsympatry with *Nv*, however, *Nv* males bite an exit hole into the puparium through which virgin *Ng* females can emerge. Similar effects could have potentially affected the outcome of the artificial sympatry experiment (chapter 6) in which *Nl* males biting exit holes into fly puparia of shared hosts could have potentially decreased the selection pressure imposed on *Nl* females by *Ng* males.

FUTURE RESEARCH

Nasonia in sympatry

To get a more complete understanding of the reproductive dynamics occurring among the *Nasonia* species, it would be worth to, first, fill the gaps in our knowledge of the different species' reproductive characteristics such as territoriality, aggression, within-host-mating, male abdominal sex pheromone and marking behaviour, or degree of male and female mate discrimination. These characteristics can be assessed easily making use of well-established behavioural bioassays. In connection with the co-occurrence of the species in nature, hypotheses on the role that these characteristics play in the reproductive isolation between species pairs can subsequently be established and tested in controlled observations of multiparasitised hosts. One simple way to get a first impression of the costs of microsympatry can be gained by assessing the reproductive output of females having emerged from multiparasitised hosts and comparison with the reproductive output of females

having emerged from hosts parasitised by one species alone. In a more advanced state, it would be also interesting to model interspecific interactions and reproductive outcomes in an agent-based modelling approach that allows to switch on and off the different reproductive isolation mechanisms and relate the outcome of simulations to data gained from observations of real wasps.

Enantioselective olfactory perception

Nasonia females respond enantioselectively to the two male sex pheromone components (4*R*,5*S*)-5-hydroxy-4-decanolide (RS) and RR (Niehuis et al., 2013). The synergistic effect of the stereoisomer RR is only observed in *Nv* females, and only *Nv* females differentiate between RS and RS supplemented with RR (Ruther et al., 2007; Steiner & Ruther, 2009a; Niehuis et al., 2013). In *Nv*, 225 odorant receptor (OR) genes have been identified in total (Robertson et al., 2010). A comparison of the ORs found in the different *Nasonia* species and sexes might be valuable as a starting point to functionally characterise the ORs that are connected to the perception of RS and RR. In consequence, this would offer the opportunity to achieve major advance in the understanding of the functioning and evolution of the perception of chiral substances in insects in general.

Phylogenetic approach to the evolution of prezygotic reproductive isolation

When intending to understand how species have diverged and how specific characteristics have evolved, it is necessary to look at the characteristics in a wider phylogenetic framework. Valuable insights could be gained by the study of the reproductive characteristics of other pteromalid wasps and compare them to the characteristics in *Nasonia*. This approach may be particularly helpful, for example, in the study of the evolution of chemical communication and, more specifically, in the study of how new chemical signals and their counterparts in olfactory perception have evolved. Pheromone production often exploits biosynthetic pathways and proteins that have been already present in the ancestral species. The biosynthesis of RR in *Nv*, for example, makes use of proteins that are similar to enzymes catalysing the deactivation of prostaglandins which serve various hormonal functions in insects (Stanley, 2006; Niehuis et al., 2013; Ruther et al., 2016). In addition, pheromone communication can evolve by a change in the ecological context in which a specific substance has been used in the past. In the parasitoid wasp *Leptopilina heterotoma* (Hymenoptera, Figitidae), for example, (-)-iridomyrmecin has evolved from a general defensive compound into both, a semiochemical cue to avoid female competition and a species-specific sex pheromone (Weiss et al., 2013). Whether males of other pteromalid

wasps also produce abdominal sex pheromones, whether they use the same substances as males of *Nasonia*, and how the used substances are biosynthesised are interesting questions to be answered in future studies.

Ecological approach to the evolution of prezygotic reproductive isolation

Another approach to the study of the evolution of prezygotic reproductive isolation mechanisms is the broadening of the scope to include other species that occur in sympatry with *Nasonia* wasps and likely interfere with them on shared host patches. Reproductive interference often occurs between closely related species, but can also happen among species belonging to different genera or even different families (McLain & Pratt, 1999; Gröning & Hochkirch, 2008; Shuker et al., 2015). Interference with any other co-occurring species thus has the potential to shape the reproductive characteristics of a species. In cattle barns, *Nv* has been found in fly puparia together with several other parasitoid wasps belonging to various different genera and families, e.g. *Muscidifurax* spp. (Pteromalidae), *Trichomalopsis* spp. (Pteromalidae), *Urolepis rufipes* (Pteromalidae), *Phygadeuon* sp. (Ichneumonidae) and *Synacra* sp. (Diapriidae; Floate et al., 1999; Gibson & Floate, 2004). In birds' nests of cavity-nesting birds, *Nv* has been found in sympatry with nine other parasitoid species from five different families (Peters & Abraham, 2010). How these species interact, whether reproductive interference occurs between them, and how reproductive interference is avoided necessitates future investigation and may give valuable insights into how species differences and reproductive isolation evolve in nature.

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